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CYTOLOGIC EFFECTS OF AIR FORCE CHEMICALS (SECOND OF A SERIES)

R. D. BENZ
R. E. RASMUSSEN
A. M. ROGERS
P. A. BELTZ
T. T. CROCKER

THE REGENTS OF THE UNIVERSITY OF CALIFORNIA
UNIVERSITY OF CALIFORNIA, IRVINE
IRVINE, CALIFORNIA 92717

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AEROSPACE MEDICAL RESEARCH LABORATORY
AEROSPACE MEDICAL DIVISION
AIR FORCE SYSTEMS COMMAND
WRIGHT-PATTERSON AIR FORCE BASE, OHIO 45433

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The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Information Office (OI) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER

ANTHONY A. THOMAS, MD
Director
Toxic Hazards Division
Aerospace Medical Research Laboratory

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The research programs of the Cytology, Cell Biology and Cytogenetics Subprogram of the Toxic Hazards Research Unit for the period July, 1978 through June, 1979 are reviewed in this report. Sister chromatid exchange and micronucleus induction in dogs, rats and mice exposed in vivo and their cells in vitro; induced gene mutations in L5178Y mouse lymphoma cells; DNA synthesis inhibition in four in vitro cell lines; and the effects on cell proliferation in mice and rats have been studied. Chemicals tested for their effects include EMS, MMS, MMC, CP, Hz, MMH, SDMH, UDMH, DMN, JP-4, JP-5, JP-10, DFM, MCH, Decalin, DMSO, ETOH and MEOH

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Monomethylhydrazine (MMH)
1,2-Dimethylhydrazine (SDMH)
1,1-Dimethylhydrazine (UDMH)
Dimethylnitrosamine (DMN)
Jet Propellent Four (JP-4)
Jet Propellent Five (JP-5)
Jet Propellent Ten (JP-10)
Decahydronaphthalene (Decalin)
Marine Diesel Fuel (DFM)
Methylcyclohexane (MCH)
Dimethylsulfoxide (DMSO)
Acetone (ACT)
Ethanol (ETOH)
Methanol (MEOH)

Ouabain (Oua)
Thymidine (Tdr)
Thioguanine (TG)
Cytosine Arabinoside (ara-C)

PREFACE

This is the third annual report of the Cytology, Cell Biology and Cytogenetics Section of the Toxic Hazards Research Program performed by the Department of Community and Environmental Medicine of the University of California, Irvine (UCI) on behalf of the Air Force under contract AF33615-76-C-5005. This report describes the accomplishments from July, 1978 through June, 1979. During this period, T.T. Crocker, M.D. served as the principal investigator, R.E. Rasmussen, Ph.D. (mouse micronucleus, DNA synthesis and cell proliferation studies) was the coordinator for the subprogram and operated the laboratory facilities at UCI, and R.D. Benz, Ph.D. (dog micronucleus studies), P.A. Beltz (rat and dog sister chromatid exchange and rat micronucleus studies) and A.M. Rogers, D. Phil. (mouse lymphoma studies) operated the laboratory facilities at the Toxic Hazards Research Unit (THRU) in Dayton, Ohio. K.C. Back, Ph.D., Chief of the Toxicology Branch was the technical monitor for the Aerospace Medical Research Laboratory.

This subprogram continues to be concerned with the detection and study of toxic effects of compounds of interest to the Air Force and other agencies as these effects may be manifested at the cellular or subcellular level in exposed animals, including humans. Among its goals are the study of chromosome abnormalities, detection of mutagenic or carcinogenic events, activation or inactivation of detoxifying enzymes, effects on DNA synthesis and repair, and effects on cell proliferation.

Activities at the THRU have been concerned with the development and application of cytogenetic test procedures for mammalian cells exposed in vitro and in vivo. Three tests, the micronucleus test, the sister chromatid exchange test, and the mouse lymphoma mutagenicity test are being used to monitor possible genetic damage caused by agents being studied.

Complementary studies are underway at UCI in which other in vivo and in vitro tests are being evaluated to determine which may be of use in the detection of genetic damage produced in experimental animals as the result of exposure to materials of military and industrial inportance.

SUMMARY

Two chemicals have been tested using the sister chromatid exchange test. The known mutagen mitomycin C (MMC) was found to give the expected positive results when tested in vitro with rat peripheral lymphocytes or bone marrow cells. The military fuel component decahydronaphthalene (decalin) did not induce sister chromatid exchanges in peripheral lymphocytes taken from dogs exposed continuously by inhalation for thirteen weeks. In vitro and in vivo exposures of rat tissues are planned to test these and other chemicals.

Many chemicals were tested using the micronucleus test. The known mutagens ethylmethanesulfonate (EMS), methylmethanesulfonate (MMS), and MMC were found to give the expected positive results when tested in vitro using canine peripheral lymphocytes. The known mutagen cyclophosphamide was found to induce micronuclei in marrow cells when injected into mice. The fuel component decalin did not induce micronuclei in peripheral lymphocytes taken from dogs exposed continuously by inhalation, nor did the military fuel JP-5 induce micronuclei in bone marrow cells taken from mice exposed by intraperitoneal injection. None of the fuels, fuel components, breakdown product, or solvents hydrazine, monomethylhydrazine (MMH), 1,2-dimethylhydrazine (SDMH), 1,1-dimethylhydrazine (UDMH), dimethylnitrosamine, JP-4, JP-5, JP-10, decalin, marine diesel fuel, methylcyclohexane, dimethylsulfoxide, dimethylsulfoxide/acetone mixture, or methanol induced micronuclei in canine peripheral lymphocytes when treated in vitro. The solvent ethanol did induce micronuclei in canine lymphocytes treated in vitro at very high dose levels. In vitro (with and without metabolic activation) and in vivo exposures of rat tissues by these chemicals are planned.

EMS, MMH (with and without metabolic activating enzymes present), UDMH, and SDMH were tested with the L5178Y mouse lymphoma gene mutation test. EMS was found, as expected, to induce mutations for ouabain, thioguanine, thymidine, and cytosine arabinoside resistance. UDMH induced resistance to thymidine and SDMH very weakly induced thioguanine resistance. All other tests were negative.

MMH was found to behave similarly to mutagens in its effect on DNA synthesis when tested with Chinese hamster ovary, Chinese hamster lung fibroblast, and two adult rat lung cell lines.

JP-5 was found not to affect cell proliferation in rats and mice exposed by inhalation.

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CYTOGENETIC STUDIES

Damage to the genetic material of a cell, the DNA molecules and their associated proteins, can be divided into two kinds. One kind is point mutations. These are small deletions of one (or a few) bases in the DNA molecule in a chromosome, or chemical changes in one (or a few) base(s) causing it (them) to pair differently when new DNA is synthesized. These result in a change in the DNA sequence which results in different information being presented for use in the cell. This can be detected by testing whether a cell loses an ability (or acquires a new ability) to metabolize a particular substrate or to be resistant to a particular drug that is harmful to normal cells.

The other kind of damage is chromosome breaks. These are gross changes in the structure of one or more chromosomes such as losses of pieces or exchanges of pieces. The difference between these two general kinds of damage is one of scale, since very large deletions in the DNA molecule indeed are losses of small chromosome pieces.

Four things can happen if a chromosome is broken: 1) The piece may be perfectly repaired at its original location. This event can be detected by monitoring DNA synthesis which occurs as part of the repair process (an unscheduled DNA synthesis assay); 2) The piece may be repaired, but during the repair process it is exchanged with the analogous piece on the other arm of the chromosome (the sister chromatid). This event can be detected by monitoring the exchange of parts of two sister chromatids which have been differentially stained (a sister chromatid exchange assay); 3) The piece may be repaired, but not at its original position, e.g. the piece from one chromosome becomes attached to a completely different chromosome. This can be detected by closely examining metaphase chromosomes for unusual morphologies (a chromosome aberration assay); 4) The piece may remain unrepaired. This can be detected in mitotic cells with a chromosome aberration assay, but the broken piece can also be detected as a small, separate nucleus in interphase cells (a micronucleus assay).

We are studying both kinds of cytogenetic damage. We are using the sister chromatid exchange test, a very sensitive assay for induced chromosome rearrangement, the micronucleus test, a very simple and rapid assay for induced chromosome breakage, and a test using L5178Y mouse lymphoma cells, a very sensitive assay for point mutations.

SISTER CHROMATID EXCHANGE TEST

INTRODUCTION

When a broken chromosome piece is exchanged for the identical piece on the other half of the same chromosome during repair, a sister chromatid exchange (SCE) has occured. A method has been developed (Latt, 1973; Perry and Wolf, 1974; Chaganti, Schonberg and German, 1974) by which one chromosome half of a given chromosome can be stained very lightly while the other half is stained darkly. This differential staining is obtained by growing dividing cells in a medium containing bromodeoxyuridine (BrdU). The cells use the BrdU in place of thymidine (one of the four normal subunits of DNA) in synthesizing new DNA. After two replicating periods, there are bromine atoms in both halves of the DNA double helix in one half of each chromosome in a cell, but only in one of the two DNA halves in the other chromosome half. When the cells are stained with a particular DNA stain* that is proportionately quenched by the presence of bromine and then counterstained with Giemsa blood stain, one can see chromosomes with one light half and one dark half when observed under a light microscope. If one or more parts of chromosome halves have been induced to exchange with their sister chromatids while growing under these conditions, however, a harlequin effect results. A darkly stained chromosome half now has a light end and vice versa. These exchanges are easily seen under the microscope. At the same time, while checking for these exchanges, chromosome aberrations can be scored.

This technique is now being used by a large number of researchers to test many different types of chemicals for the ability to induce chromosome rearrangements in many different types of cells after both in vitro or in vivo exposure. It is also being evaluated for its ability to monitor human exposure experience by testing blood samples of humans who work under hazardous conditions (Carrano, 1978; Lambert et al., 1978; Kucerova, Polikova and Batora, 1979).

We have used the SCE test with dog and rat peripheral lymphocytes and rat bone marrow to test several chemicals with in vivo and in vitro exposures.

MATERIALS AND METHODS

Canine Peripheral Lymphocytes

The materials and methods used for the SCE test with canine peripheral lymphocytes are the same as those described previously (Crocker, Benz and Rasmussen, 1978).

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* The stain is Hoechst 33258 (Hoechst) or, chemically, 2-[2-(4-hydroxyphenol)-6-benzimidazolyl]-6-(1-methyl-4-piperazyl)-benzimidazole.

Rat Peripheral Lymphocytes

Fisher 344 female rats, obtained from Charles Rivers Breeding, are anesthetized with 40 mg/kg body weight sodium pentobarbital (Abbott). Blood samples are then taken with sterile needles from an exposed subclavian vein by a method demonstrated by Nemenzo* into syringes containing 60 u of heparin (Sigma). [Taking blood by this method enables us to avoid sacrificing any animals. In this way we will be able to take serial samples from the same animal during long term in vivo exposures. It is also our goal to use methods that can be applied directly to humans.]

One-tenth milliliter of freshly-drawn whole blood is cultured in 5 ml of fresh McCoy's 5A cell medium (GIBCO) containing 30% fetal calf serum (GIBCO) for extra nutrients, 50 u/ml penicillin (Sigma) and 500 µg/ml streptomycin (Sigma) to retard bacterial contamination, and 0.05 ml leucoagglutinin (Pharmacia) to stimulate lymphocyte division, at 37° C, 95% relative humidity, and 7% CO_2 atmosphere for 72 hr. After this time, when the cells are growing well, the cell medium is changed (but without leucoagglutinin) and BrdU is added to make the mixture 10 µM BrdU. If the cells are to be treated in vitro with a chemical, it is also added at this time. The cells are then incubated for 25 more hr. After this time, when many cells are about to enter mitosis for the second time, colchicine (Sigma) is added to make the final concentration 1.2×10^{-6} M colchicine, and the incubation is continued for 4 hr additional while the mitotic cells accumulate. At this point, the culture tubes are centrifuged at 280 g for 10 min and the cell medium is removed from the cell pellet. Five milliliters of 75 mM KCl (37° C) are added slowly with gentle mixing to the cell pellet and then left to stand for 10 min. The red cells swell and explode under this treatment and are removed from the population. The white cells swell, but do not break, and the chromosomes within spread. The cells are then centrifuged and the supernatant removed. Seven milliliters of a 3:1 mixture of ice cold ethanol and acetic acid ("fix") are added to the cell pellet with prompt, thorough, but gentle mixing and then left to stand overnight at 4° C. After this, the cells are again centrifuged and then they are dropped from a 2 cm height (to smash the spherical cells flat) onto cold, alcohol washed slides that have been dipped into ice cold water. After the slides have been allowed to dry thoroughly, they are stained for 12 min with 50 µg/ml Hoechst 33258 under bright cool white fluorescent illumination and then are covered and sealed with rubber cement and exposed to intense illumination for at least an additional 3 hr. After this time, the slides are unsealed and are stained with 3% Giemsa blood stain (Searle) for 10 min, rinsed, and then permanently sealed under coverslips.

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* Personal communication

The slides are scanned under 1000 x magnification for properly stained, well spread metaphase chromosomes. The total number of exchanges seen per the total number of chromosomes scored are tabulated and averages are calculated for each dose level. We have defined a positive response as a dose dependent increase in the number of SCEs per cell that is statistically greater than twice the measured control level.

Rat Bone Marrow

Using rats of the type listed in the previous section which have been anesthetized as stated, marrow is taken by inserting a spinal needle (Yale) into the femur of the animal using a technique described by Nemenzo, Pasi, and Hine (1975). [Again no animals are sacrificed.] The marrow is allocated from the syringes which contain 60 u heparin by placing 0.076 ml into culture tubes containing cell medium made up as specified and cultured under the conditions previously described. After culturing the cells for 6 hr, the supernatant cell medium is removed and fresh medium is added. In addition at this time BrdU is added to make the mixture 10 μM BrdU. If the cells are to be treated in vitro with a chemical, it is also added at this time. The cells are then incubated for an additional 15 hr after which time many cells are about to enter mitosis for the second time. Colchicine (1.2×10^{-6} M) is then added for 6 hr and then all subsequent steps are identical to those used for peripheral lymphocytes, except that KCl is added for 20 min at 37^{o} C (instead of 10 min).

RESULTS

Canine Peripheral Lymphocytes Exposed in Vivo

Blood samples were taken from dogs exposed continuously by inhalation to decahydronaphthalene (decalin, Dupont) (MacEwen and Vernot, 1978). Figure 1 shows a well spread canine peripheral lymphocyte stained to show sister chromatid exchanges. This cell was obtained from an animal that had not yet been exposed to decalin. Eleven exchanges can be seen (three exchanges is a more typical spontaneous rate). Figure 2 shows that exposure by inhalation to decalin at the stated concentrations does not induce a significant number of SCEs above the control value in canine lymphocytes.

Rat Peripheral Lymphocytes and Bone Marrow Cells Exposed in Vitro

Rat blood and marrow samples were exposed in vitro to various doses of mitomycin C (MMC, Sigma), a well known bifunctional DNA alkylating agent. Figure 3 shows typical metaphase spreads of a rat lymphocyte and a rat bone marrow cell stained to show sister chromatid exchanges. The induction of SCEs in rat lymphocytes and bone marrow cells by MMC is shown in Figure 4. An obvious positive response can be seen in both instances. Note that a dose lower than 10 nM of MMC can be seen to be positive. An experiment to confirm these results with MMC is currently underway and other chemicals of interest, many of which have never before been tested in this way, will soon be tested.

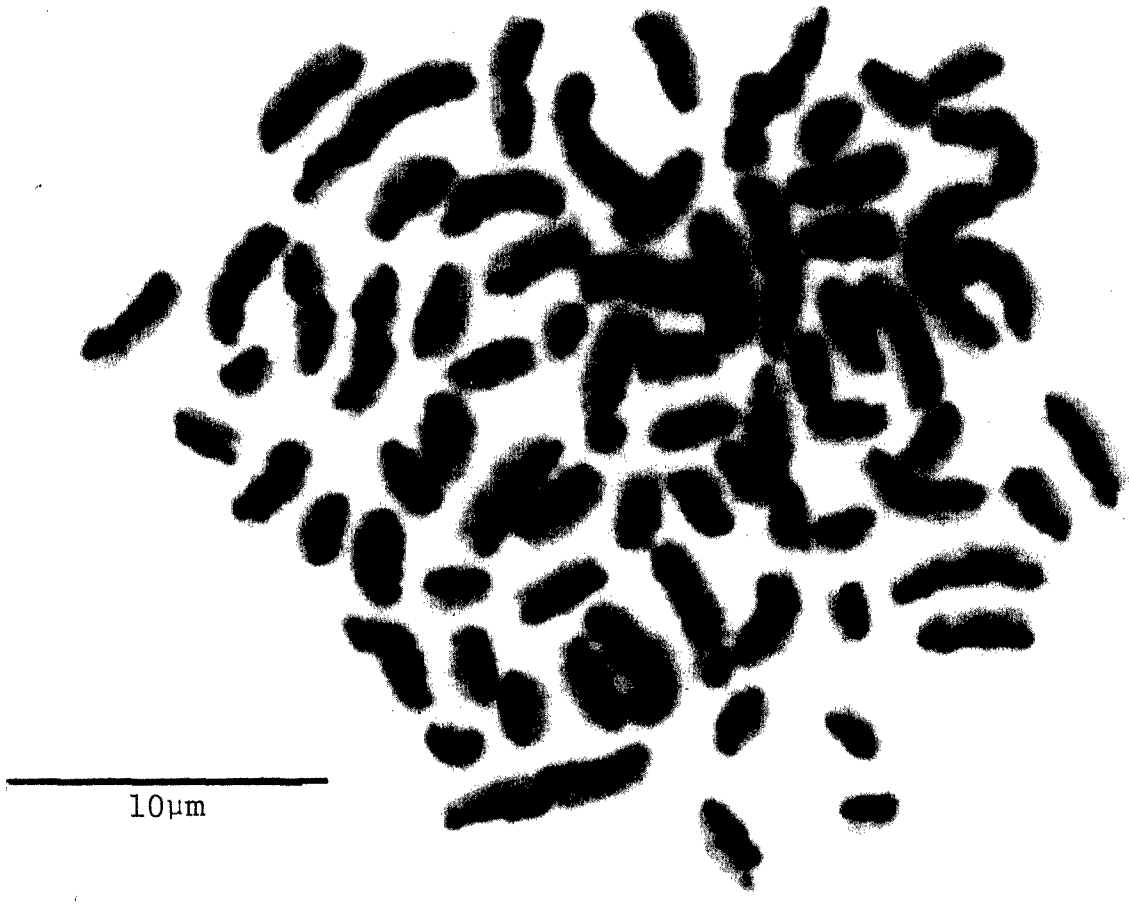


Figure 1. Canine peripheral lymphocyte metaphase chromosomes stained to show SCEs.

After this, the next step in using the SCE test will be to expose rats in vivo to various chemicals and observe the induction (or lack thereof) of SCEs. The first exposure series will be to MMC with serial peripheral blood and marrow samples taken from the same group of animals to monitor long term effects. This study is designed essentially to repeat the work done by Stetka, Minkler and Carrano (1977) who used peripheral blood obtained from rabbits. Other chemicals of interest will then be tested.

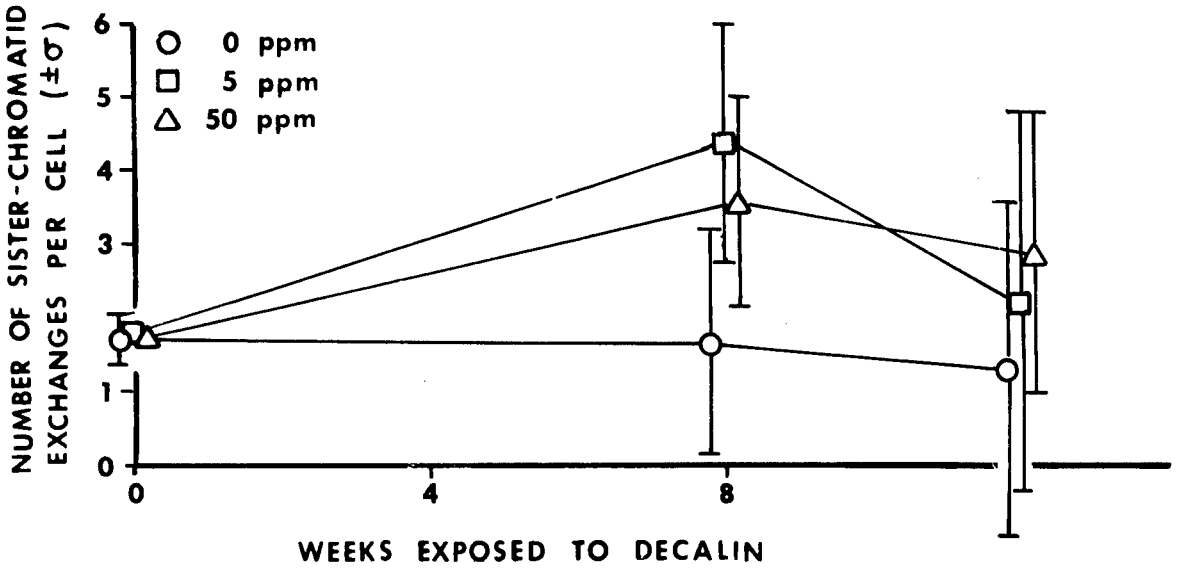


Figure 2. Induction of SCEs in canine peripheral lymphocytes after in vivo exposure by inhalation to decalin.

DISCUSSION

The SCE assay is by far the most sensitive assay of chromosome damage now developed in mammalian systems (Perry and Evans, 1975). It is as much as one thousand-fold more sensitive than assays where broken chromosome pieces (chromosome aberrations of micronuclei) are scored. It is therefore worthwhile to attempt to develop this assay even though it requires obtaining well spread mitotic chromosomes and involves tedious scoring routines.

Successfully prepared canine chromosome preparations have been obtained for statistically useful results on only four occasions, however. The first of these was while testing blood samples from dogs that had been exposed by inhalation to marine diesel fuel (DFM) vapor continuously for thirteen weeks (Crocker, Benz, and Rasmussen, 1978). The other successful results were obtained with samples from dogs that were exposed continuously for three different times to decalin. We have discussed the problems involved in using the SCE test with this species previously (Crocker, Benz, and Rasmussen, 1978). We have tried several modifications of the method stated for obtaining properly spread and stained canine chromosomes since our previous report (methods suggested by our experience with rat chromosomes) and we have obtained improved results. However, we still feel that the method does not produce scoreable canine chromosomes with enough efficiency for it to be used routinely. We will therefore not do posi-

Figure 3. Metaphase chromosomes stained to show SCEs in (top) a rat peripheral lymphocyte and (bottom) a rat bone marrow cell.

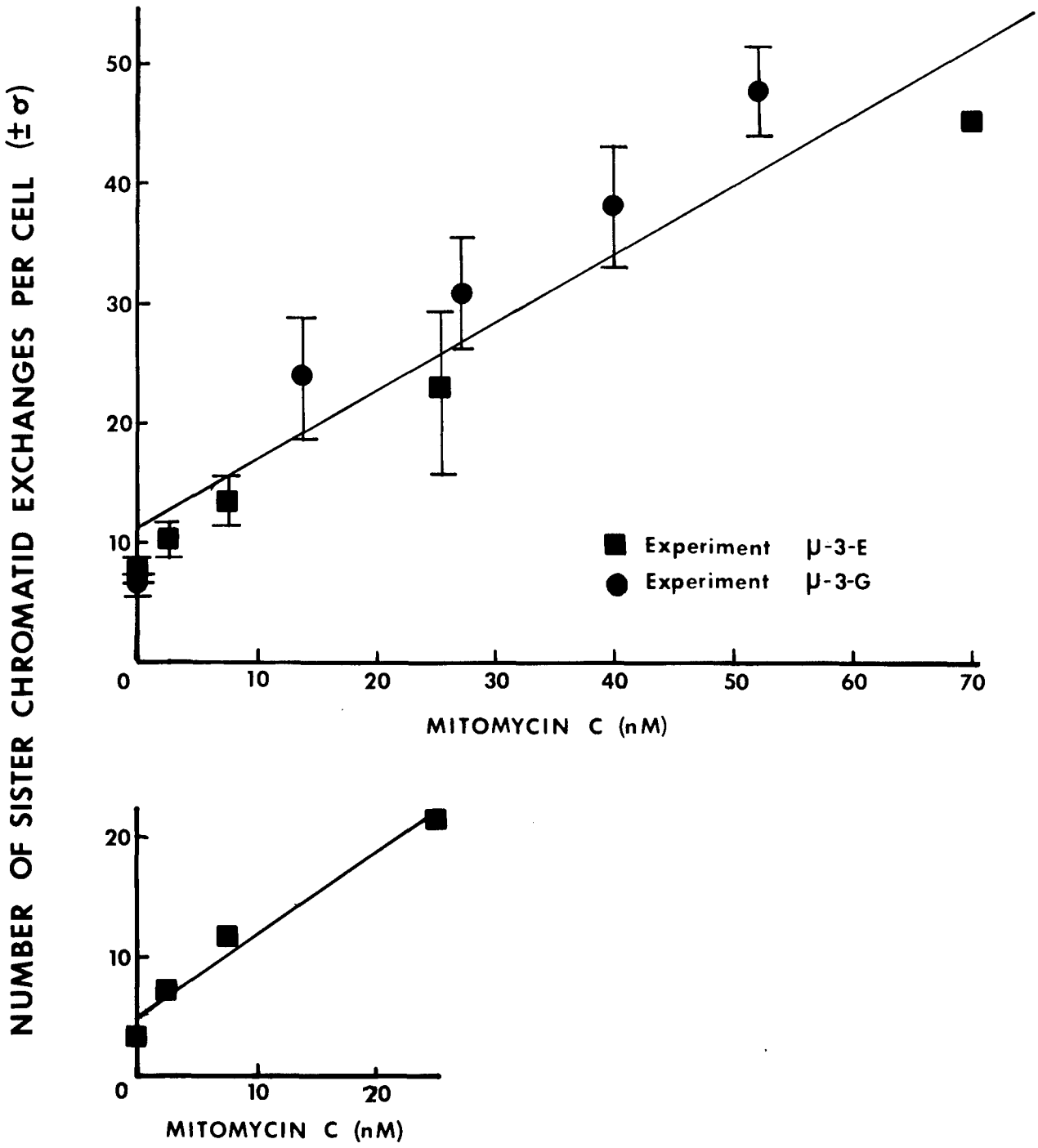


Figure 4. Induction of SCEs in (top) rat peripheral lymphocytes and (bottom) rat bone marrow cells after in vitro exposure to MMC.

tive controls for the dog inhalation studies we have reported, but we will do positive controls in our work using rats.

We now have very successful methods for obtaining on a regular basis well spread rat peripheral lymphocyte and bone marrow chromosomes. We have begun to use these methods to test a variety of chemicals by in vitro and in vivo intraperitoneal (i.p.) injection and inhalation exposures. We intend to establish the sensitivity of the SCE test with rats and because the testing will be done without sacrificing any animals, enabling us to take repeated samples from the same animal, we will also be able to compare the results of this short term test to that of traditionally conducted long term toxicologic tests using the same animals so that a direct comparison can be made.

MICRONUCLEUS TEST

INTRODUCTION

It is possible to stop dividing mammalian cells in metaphase with the drug colchicine. After preparation for microscopic examination, mitotic chromosomes of cells can be examined and breaks observed directly. This procedure, however, is tedious since it is difficult to prepare cells with metaphase chromosomes that are well spread out on the microscope slide. A technique has been developed, however, that overcomes this problem (Heddle, 1973; Countryman and Heddle, 1976; Countryman and Heddle, 1977; Heddle, Benz, and Countryman, 1978; Heddle, Lue, Saunders, and Benz, 1978). Instead of arresting the cells in metaphase, lymphocytes are allowed to continue into the next interphase. If a broken chromosome piece is present in metaphase, quite often this piece will not be drawn into the nucleus at the next interphase since there is no spindle attached. Instead, this piece will form its own "micronucleus." Thus, to assay unrepaired chromosome breaks in lymphocytes, interphase cells can be stained and scored for the presence of micronuclei.

Alternately, immature bone marrow erythrocytes can be examined. This assay is based on the observation that chromosomal damage during maturation of erythrocytes may result in acentric fragments that are not extruded at the normal point in the maturation process but persist in the erythrocytes as micronuclei. These micronuclei can be detected and quantified by appropriate staining methods.

The micronucleus test is not as sensitive as the SCE test, but it is so much easier to do that where dose levels are not limited, it is the test of choice (Raj and Heddle, 1979). It has also been shown that while DNA breakage is involved in both SCE and micronucleus production, the pathways for these end results are not identical (Galloway and Wolff, 1979). Some chemicals have been found to increase the number of SCEs, but not micronuclei, and other chemicals do the opposite (although most chemicals tested either increase both or neither). Therefore, it is valuable to do the micronucleus test in addition to the SCE test because different types of damage are involved.

We have used the micronucleus test with dog and rat peripheral lymphocytes and have just begun to use rat and mouse bone marrow to test a variety of chemicals with in vitro and in vivo exposures.

MATERIALS AND METHODS

Canine Peripheral Lymphocytes

The materials and methods reported previously (Crocker, Benz, and Rasmussen, 1978) were used for the micronucleus tests with canine peripheral lymphocytes exposed both in vivo and in vitro reported here.

Rat Peripheral Lymphocytes

Blood samples are obtained from rats as described in the materials and methods of the SCE section of this report.

Freshly drawn whole blood (0.02 ml) is cultured in 1 ml McCoy's 5A cell medium containing 30% fetal calf serum, 50 u/ml penicillin, 500 µg/ml streptomycin, and 0.013 ml leucoagglutinin, at 37° C, 95% relative humidity, and 7% CO_2 atmosphere for 76 hr, after which the cell medium is replaced with fresh McCoy's 5A made as above except no leucoagglutinin is added. If the cells are to be treated with a chemical, this is also added at this time. The cells are then incubated for an additional 24 hr. After this time, the culture tubes are centrifuged at 12,000 g for 15 sec and the cell medium is removed from the cell pellet. One milliliter of 75 mM KCl is added to the cell pellet and left to stand for 12 min. The cells are then centrifuged and the supernatant removed. One milliliter of a 3:1 mixture of ethanol and acetic acid ("fix") is added to the cell pellet with gentle but thorough mixing and then left to stand for 10 min. The cells are then centrifuged and most of the supernatant is removed. The cell pellet is resuspended thoroughly in the remaining supernatant and then dropped from a 7 cm height onto alcohol washed slides that have been dipped into water. After the slides have been allowed to dry, they are stained with 3% Giemsa blood stain for 10 min, rinsed, and then permanently sealed under coverslips. The slides are then examined under 400 x magnification for the presence of nuclei with associated micronuclei. At least five hundred nuclei and the total number of micronuclei seen with them are scored for each dose level in each slide, and the average of two slides is calculated and reported as the number of micronuclei per 1000 cells. We have defined a positive response as a dose dependent increase in the number of micronuclei per cell that is statistically greater than twice the measured control level.

Rat Bone Marrow

Bone marrow samples are obtained from rats as described for the SCE test. Using the same cell medium mixture and under the same conditions as are described for rat lymphocytes, 0.038 ml of freshly drawn marrow is cultured for 5 hr after which the cell medium is changed and any chemical to be tested is added. Then the cells are incubated for an additional 24 hr. After this time the culture tubes are centrifuged at 12,000 g for 15 sec and the cell medium is removed

from the cell pellet. The mixed pellet is then put onto alcohol washed microscope slides and spread with coverslips. After drying overnight, the slides are treated for 5 min with methanol and then allowed to dry once again. They are then stained for 45 min with 5% Giesma blood stain, rinsed, dried, and then permanently sealed under coverslips. The slides are then examined for the presence of micronuclei within immature polychromatic erythrocytes. Data are handled as described for rat lymphocytes.

Mouse Bone Marrow

Bone marrow from both femurs is flushed into fetal calf serum and smears are prepared and stained with Giesma blood stain as described for rat bone marrow. Mature erythrocytes stain pinkish, and immature ones stain with a bluish cast. Micronuclei appear as refractile, circular, dark bodies in both mature and immature erythrocytes. Counts are presented only for immature erythrocytes because these must have been formed during the treatment period.

Experiment 1: Female strain BC3f1 mice approximately 9 mo old were treated i.p. with cyclophosphamide (CP) at 25 μg/gm body weight (b.w.) at 30 and 6 hr before sacrifice. Controls received saline.

Experiment 2: An experiment similar to the above was done, except that an additional mouse was treated i.p. with JP-5, (U.S. Navy) at 100 mg/kg b.w. in corn oil. The control received corn oil only. A third mouse was treated with CP as above.

Experiment 3: Studies were done with C57Bl/6 mice to establish their response to CP and to compare the response to that found with other strains of mice. The animals were treated with various doses of CP as above at 30 and 6 hr before killing.

RESULTS

Canine Peripheral Lymphocytes Exposed in Vivo

Figure 5 shows a typical canine peripheral lymphocyte nucleus with an associated micronucleus.

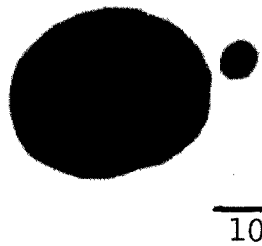


Figure 5. Canine peripheral lymphocyte nucleus and micronucleus.

Dogs were exposed by inhalation to decalin for thirteen weeks (MacEwen and Vernot, 1978). Blood samples were taken from these dogs before the exposure began and after 8 and 12 wk of continous exposure to 0, 5 or 50 ppm of decalin. The 8 and 12 wk samples were analyzed for the presence of micronuclei. Because the results of these analyses were negative (Figure 6), the preexposure sample was not evaluated. We have not yet done a positive control for in vivo canine exposures.

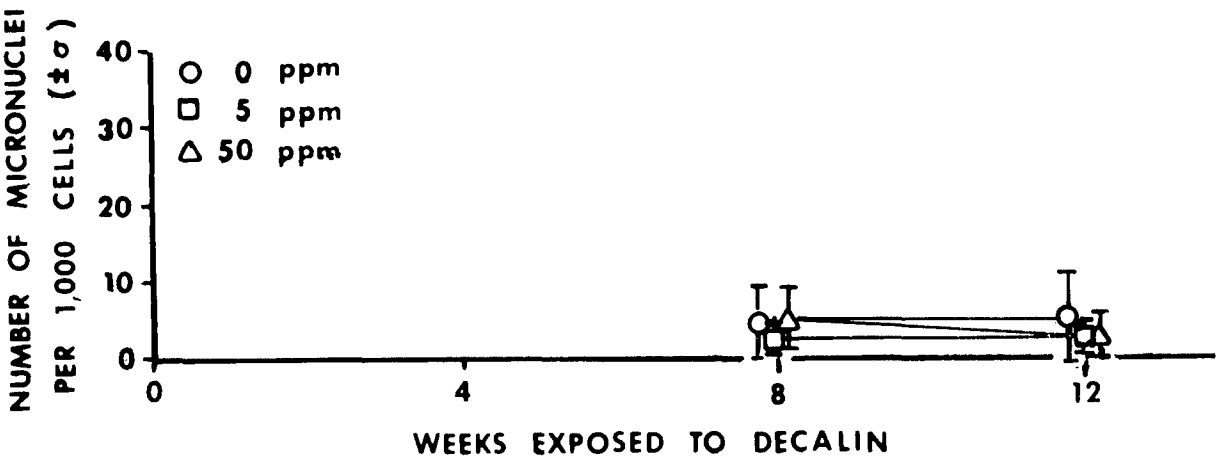


Figure 6. Induction of micronuclei in canine peripheral lymphocytes after in vivo exposure by inhalation to decalin.

Canine Peripheral Lymphocytes Exposed in Vitro

Peripheral blood samples were obtained from unexposed dogs and exposed in vitro to several chemicals. The induction of micronuclei in lymphocytes by ethylmethanesulfonate (EMS, Eastman), methylmethanesulfonate (MMS, Eastman), monofunctional DNA alkylating agents and well known mutagens, are shown in Figures 7 and 8, respectively. Figure 9 shows the induction of micronuclei by MMC, a bifunctional alkylating agent. These three chemicals serve as positive controls for the in vitro exposures. In all three figures, the general shape of the curve is due to an increasing number of chromosome breaks being caused by increasing chemical doses, followed by a decreasing number of micronuclei detected at even higher dose levels. For a cell to have a micronucleus, at least one cell division must occur. At the highest doses, the chemicals prevent cell division and therefore fewer micronuclei are seen even though, presumably, increasing chromosome breakage occurs.

Canine lymphocytes were exposed in vitro to hydrazine (Hz, Eastman), monomethylhydrazine (MMH, Eastman or Olin), 1,2-dimethylhydra-

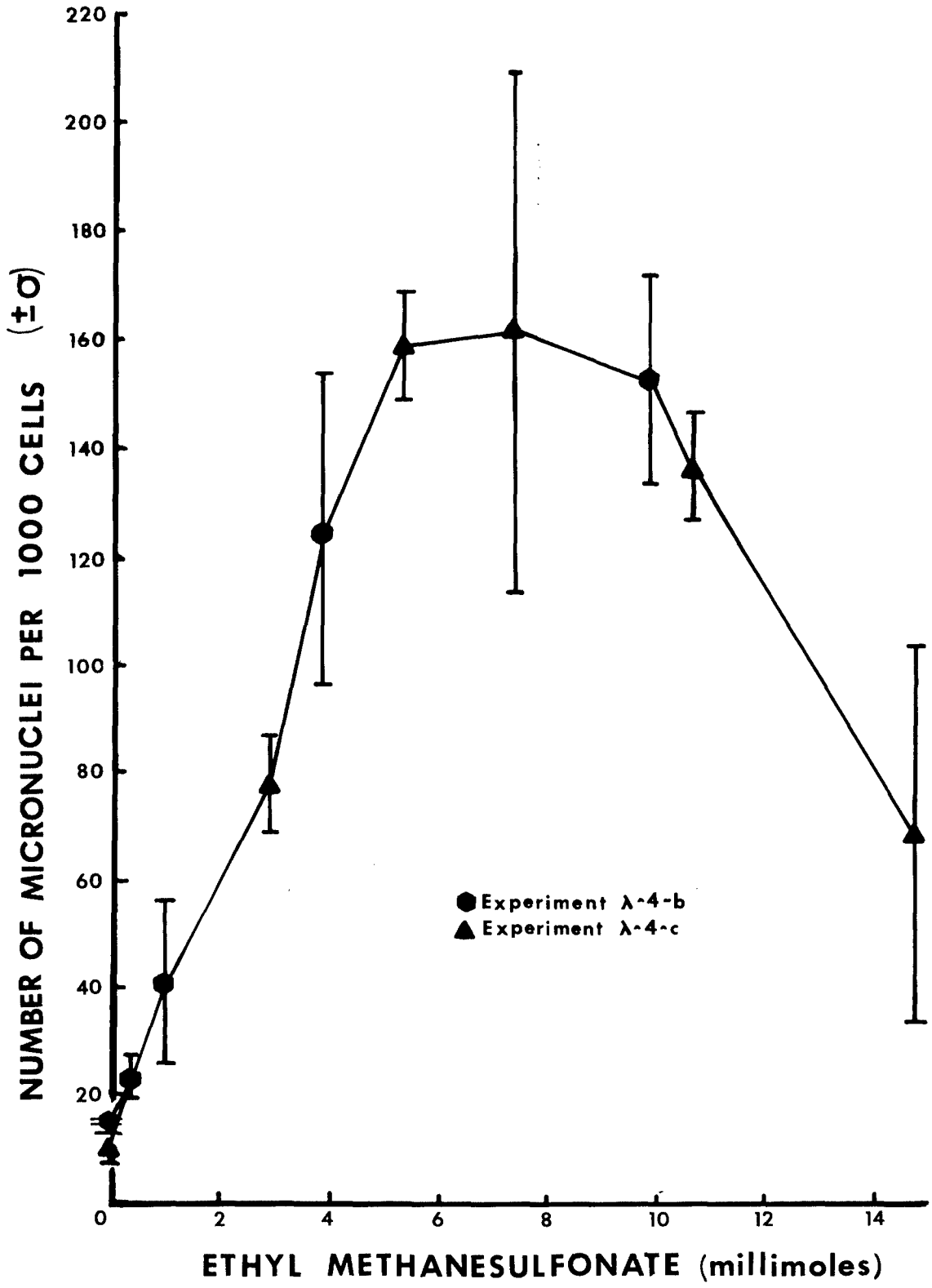


Figure 7. Induction of micronuclei in canine peripheral lymphocytes after in vitro exposure to EMS.

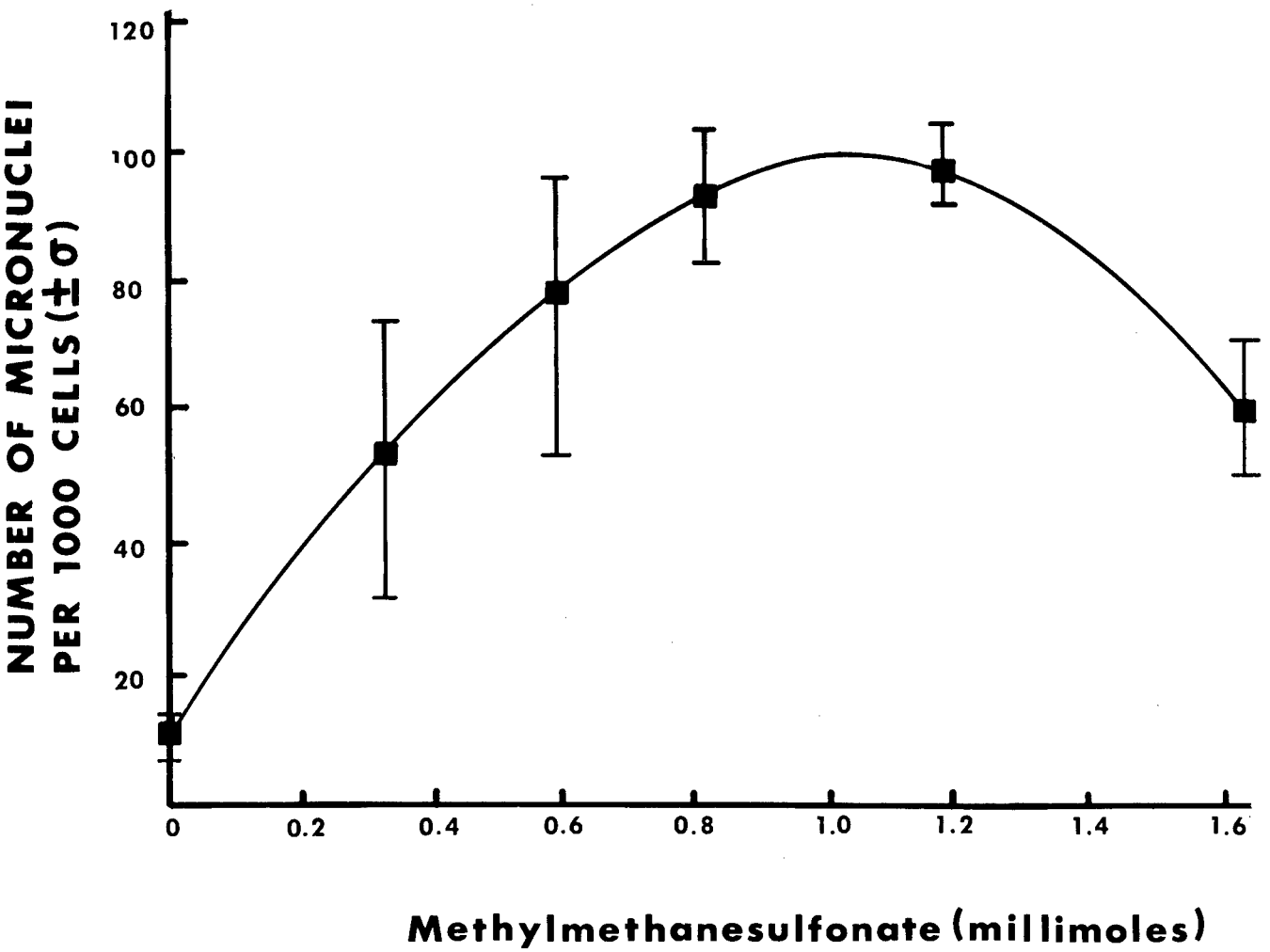


Figure 8. Induction of micronuclei in canine peripheral lymphocytes after in vitro exposure to MMS.

zine (SDMH, MC/B), 1,1-dimethylhydrazine (UDMH, U.S. Air Force), and a breakdown product of the latter, dimethylnitrosamine (DMN, Aldrich). Results of these exposures were all found to be negative even when tested at dose levels that were extremely toxic to the cells (Figure 10.)

All chemicals were added to the cell cultures at the beginning of the cell culture time, before the cells had begun to divide. Since the hydrazine compounds are relatively unstable, this could have posed a problem if the "critical time" of their effect were later in the cycle of the cells, but the hydrazines were no longer in active form. As an indication of how long the hydrazine compounds remain active, we grew L5178Y mouse lymphoma cultures in cell medium containing MMH or

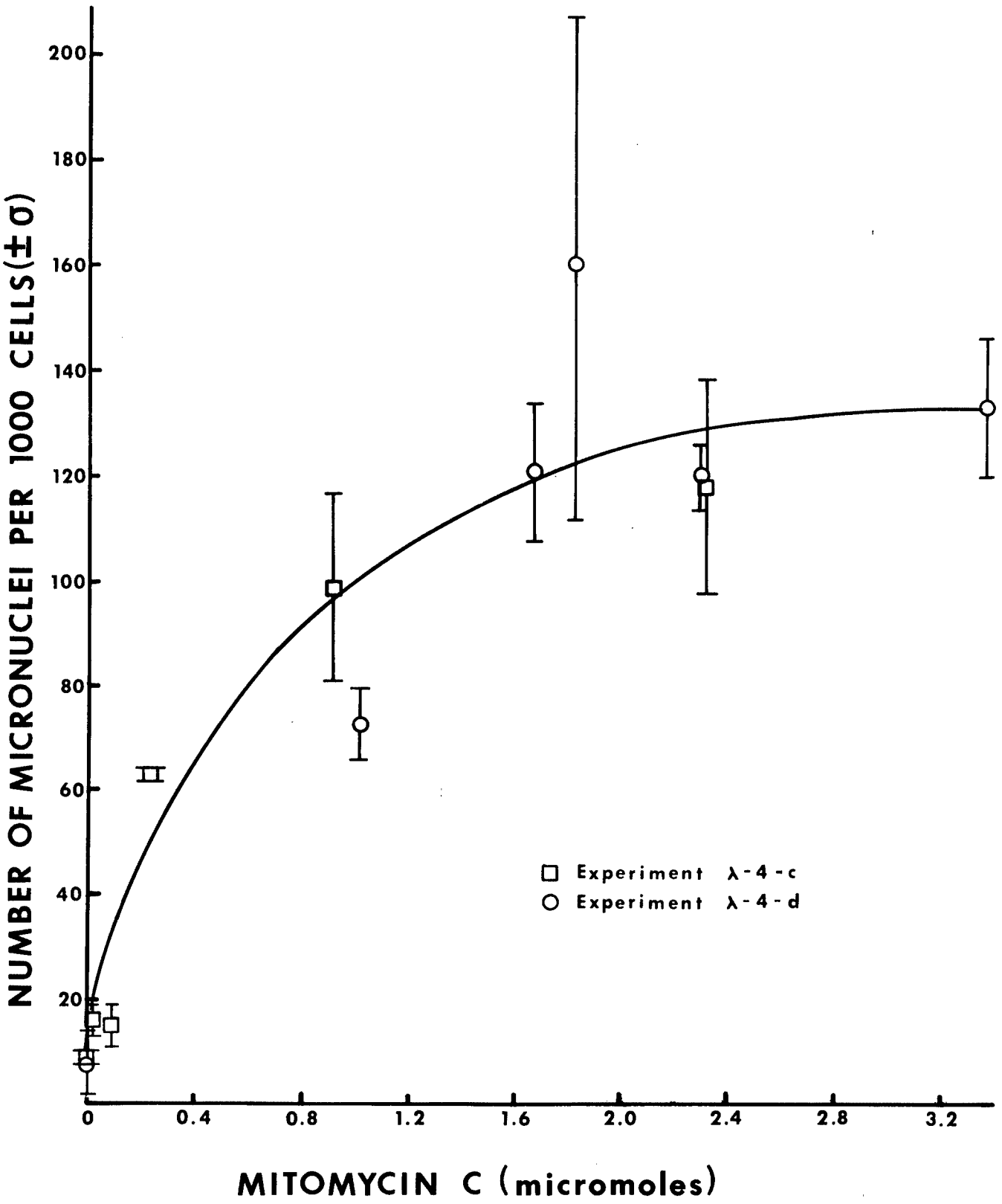
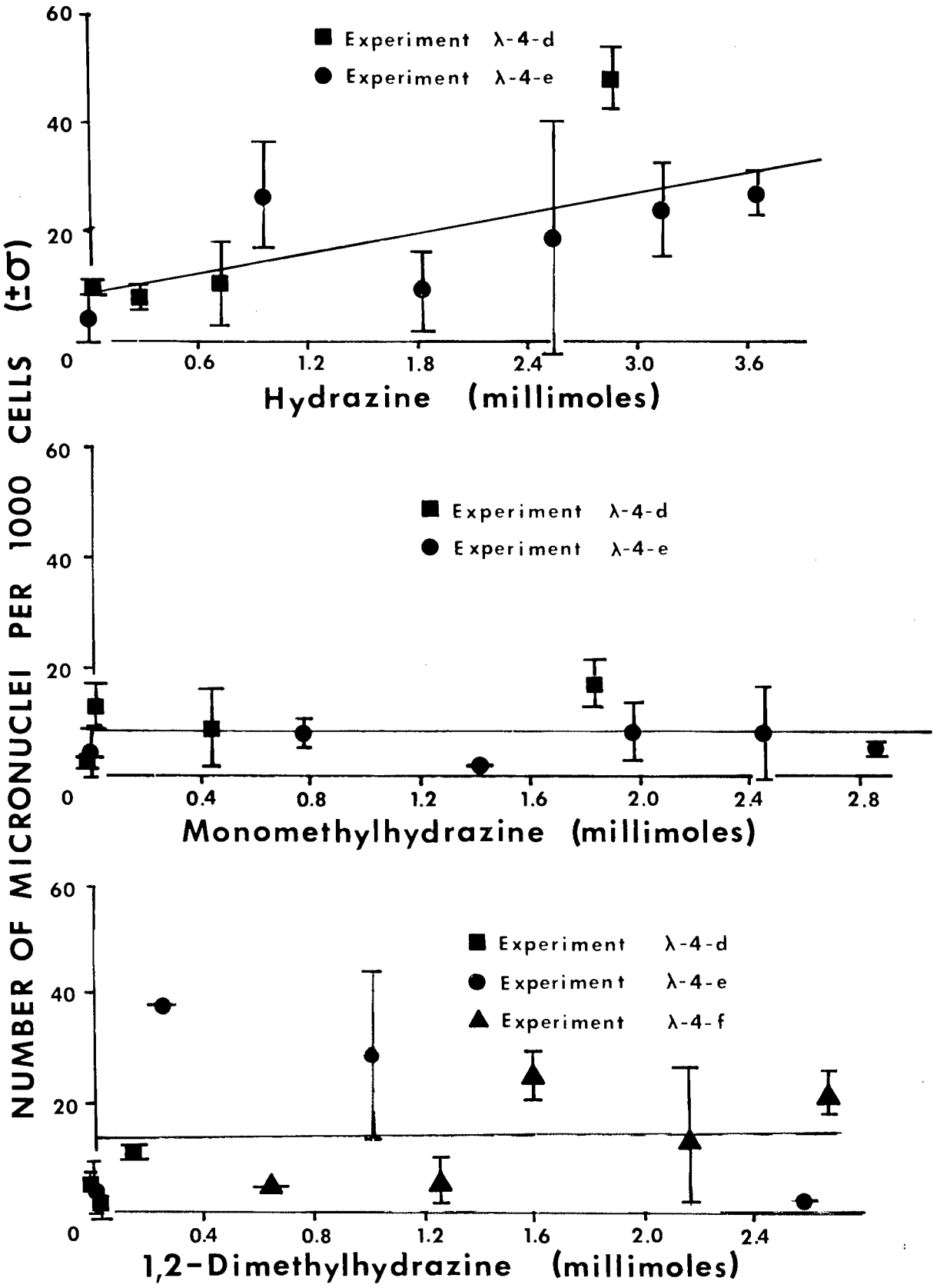


Figure 9. Induction of micronuclei in canine peripheral lymphocytes after in vitro exposure to MMC.


NUMBER OF MICRONUCLEI PER 1000 CELLS (±σ)
60
40
20
0
Experiment λ-4-d
Experiment λ-4-e
0.6
1.2
1.8
2.4
3.0
3.6
Hydrazine (millimoles)
Experiment λ-4-d
Experiment λ-4-e
0.4
0.8
1.2
1.6
2.0
2.4
2.8
Monomethylhydrazine (millimoles)
Experiment λ-4-d
Experiment λ-4-e
Experiment λ-4-f
0.4
0.8
1.2
1.6
2.0
2.4
1,2-Dimethylhydrazine (millimoles)

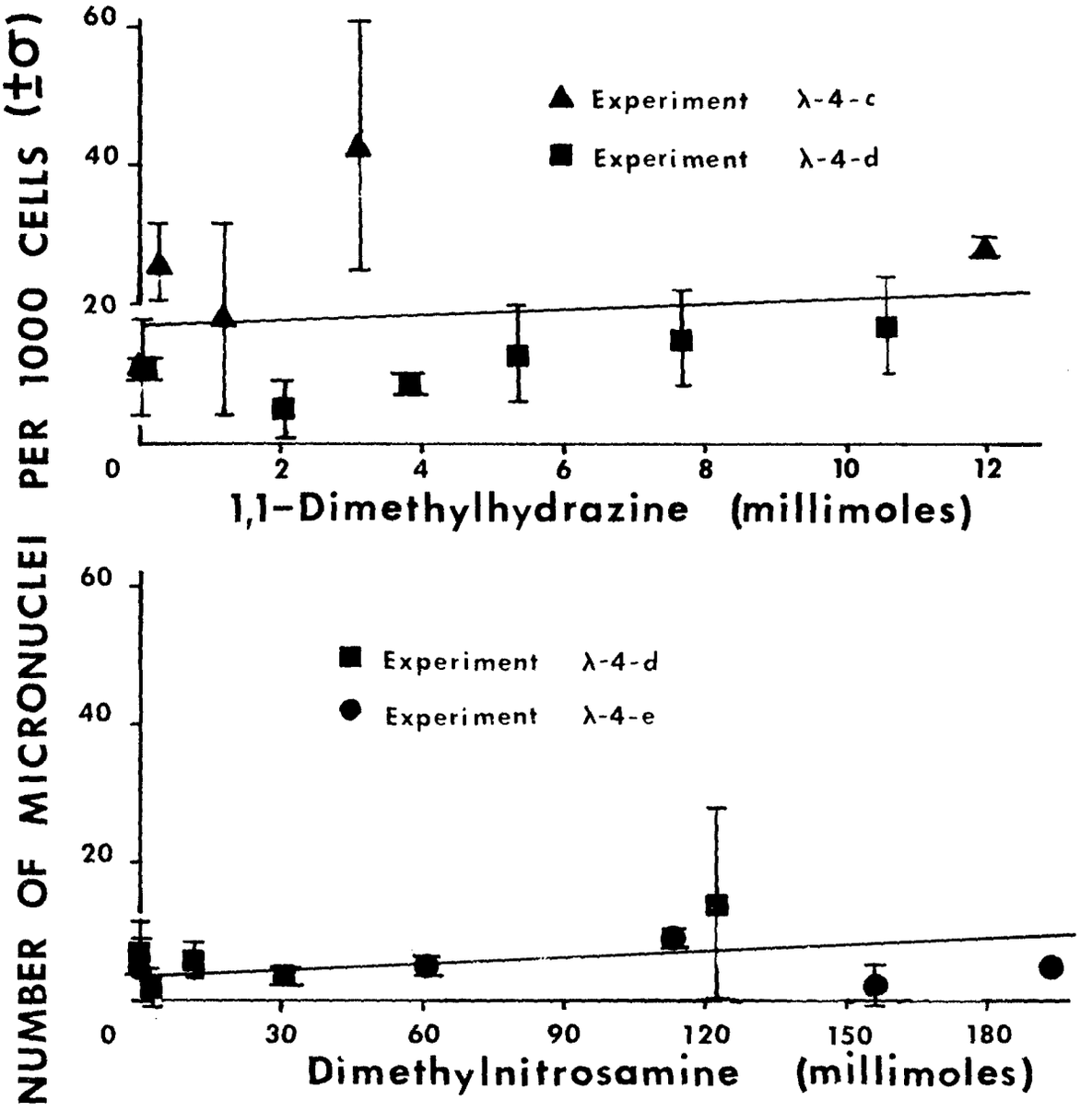


Figure 10. Induction of micronuclei in canine peripheral lymphocytes after in vitro exposure to (top, previous page) Hz, (middle, previous page) MMH, (bottom, previous page) SDMH, (top, this page) UDMH, or (bottom, this page) DMN. Lines drawn by linear regression method.

UDMH that had been previously incubated under normal cell culture conditions for 24 or 48 hr, but without cells present. Taking toxicity to the cells as the endpoint, we found that MMH and UDMH are essentially just as active after even 48 hr of preincubation in cell medium as they are when added fresh (see the section in this report concerning mouse lymphoma cells for further details and comments). We therefore believe that the hydrazines are active throughout most, if not all, of

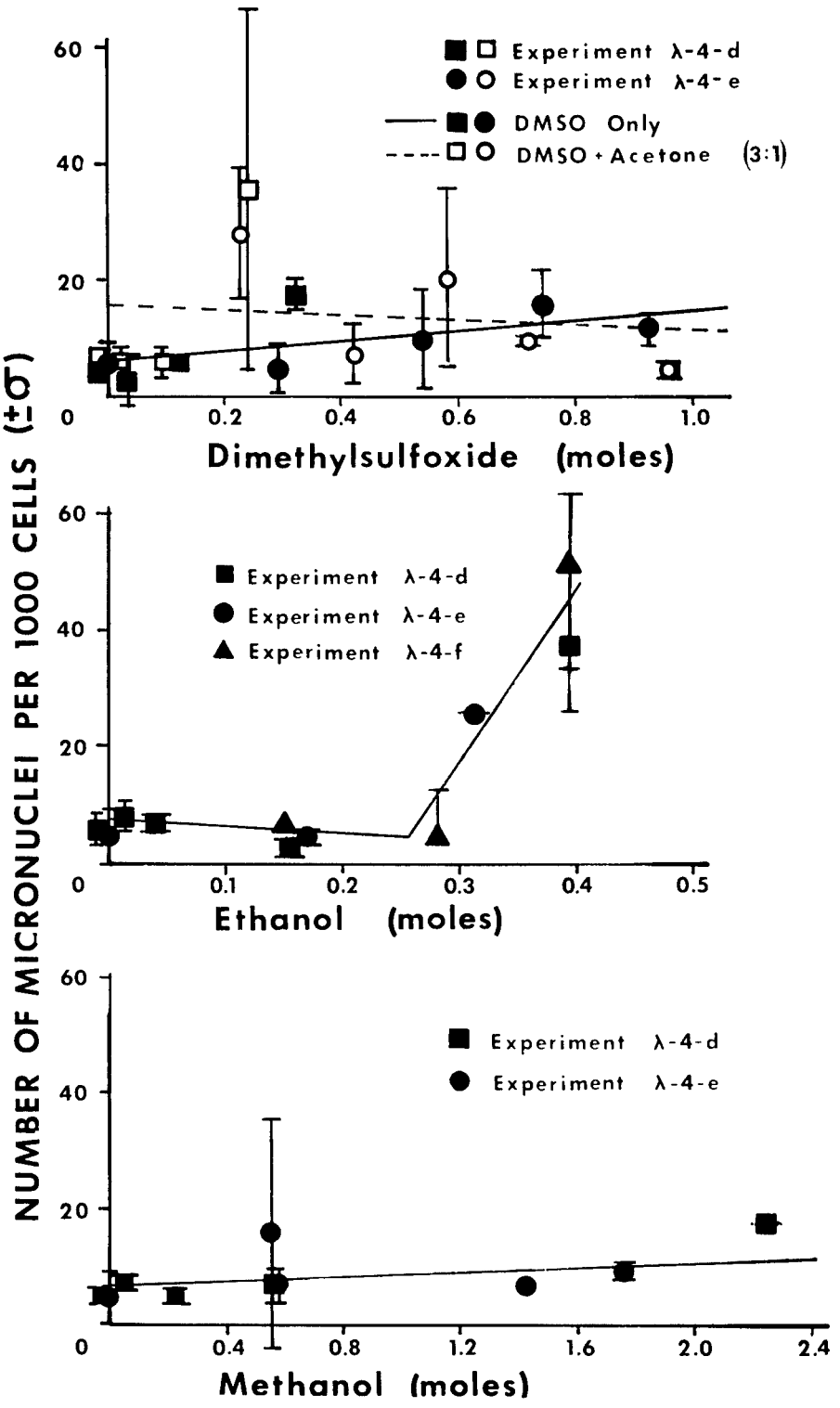


Figure 11. Induction of micronuclei in canine peripheral lymphocytes after in vitro exposure to (top) DMSO or DMSO + ACT, (middle) ETOH, or (bottom) MEOH. Lines drawn by linear regression method.

the time that canine lymphocytes are exposed to it and so if there is indeed a "critical time" for exposure, it has probably been covered.

We also intended to expose canine lymphocytes to other chemicals of military interest, specifically, jet and ship fuels. These chemicals and mixtures, however, were found to be insoluble in water, which is the major component of our cell growth medium. We therefore tested several solvents for their ability to induce micronuclei, hoping to find one of them that had no effect by itself, but could serve as an intermediary between the fuels and the cell medium. We tested dimethylsulfoxide (DMSO, Eastman), a 3:1 mixture of DMSO and acetone (ACT, Mallinckrodt), ethanol (ETOH, Mallinckrodt), and methanol (MEOH, Mallinckrodt). These chemicals were tested at up to dose levels that were seen to be extremely toxic to the cells. All solvents were found to be negative in the micronucleus test, except that ethanol was repeatedly found to be positive at very high dose levels (Figure 11). Methanol was negative at even an eight times higher dose level than the ones at which ethanol was found to be positive.

In the end, however, we found that none of these solvents served their originally intended purpose of solubilizing the fuels in water. We therefore simply added the fuels and fuel components jet propellents four, five and ten (JP-4, JP-5, and JP-10, respectively, U.S. Air Force, U.S. Navy, and Suntech, respectively), decalin, DFM, and methylcyclohexane (MCH, Eastman) to culture tubes containing cells and medium and inverted them from time to time hoping that, although it would be unquantifiable, some physical contact would be made between the lipid layer of the membranes of the cells, in which some of the fuel might dissolve. We also thought that during the 96 hr incubation period, any water soluble component of the fuels would reach the cells. After exposing the cells to 1% and 10% mixtures of the fuels in the cell medium in this way, we found no induction of micronuclei above background (data not shown). We are now investigating other means of more quantifiably exposing cells in culture to water insoluble chemicals.

Rat Peripheral Lymphocytes and Bone Marrow Cells Exposed in Vivo and in Vitro

We are currently beginning a series of experiments in which rat peripheral lymphocytes and bone marrow cells are exposed in vitro to a number of chemicals. We have no complete results to report at this time, however. We will also soon be examining for induced micronuclei cells taken from rats that are exposed in vivo.

Mouse Bone Marrow Cells Exposed in Vivo

The results of these experiments are shown in Table 1. on the basis of these results, CP appears to be an appropriate positive control substance which can be used in studies of micronucleus formation as it may be produced by environmental agents. The results also indicate that at the dosage tested, JP-5 does not induce micronucleus formation. Higher dosages have not been tested at this time.

Table 1. Micronucleus formation in mice exposed to CP or JP-5.*

Experiment Number	Control	CP Treated	JP-5 Treated
1	2.9	6.4	
2	1.7	2.7	0.4
3	1.0 1.2	2.2 2.6 (10 mg/kg)	
		6.0 5.0 (50 mg/kg)	
		4.8 3.8 (100 mg/kg) 4.0	

*Values are the percentage of immature erythrocytes containing one or more micronuclei, and are based on counts of at least 500 cells.

The results with these strains of mice are in approximate agreement with the results found by other investigators (Schmid, 1976). These results also agree with our studies reported last year (Crocker, Benz and Rasmussen, 1978) in which lymphocytes in blood samples taken from dogs exposed by inhalation to JP-5 did not contain micronuclei over control values and with our studies, presented in this report, in which JP-5 was found to not induce micronuclei when mixed with canine lymphocytes in vitro.

DISCUSSION

In vivo tests using dogs exposed to decalin or mice exposed to JP-5 were negative. In vitro tests using canine lymphocytes exposed to known clastogenic chemicals give the expected positive response in the micronucleus test. The water soluble (hydrazines) and water insoluble fuels and fuel components tested proved to be negative in this test. This included the known clastogenic chemical DMN, a breakdown product of UDMH. It has been found by many others (including Heddle and Bruce, 1977), however, that DMN is negative in all in vitro testing if not metabolically activated with liver enzymes (the S9 fraction). We plan to retest all chemicals that were found to be negative in the unactivated in vitro tests with an S9 fraction present to test the potency of the chemicals when activated.

As with the SCE test, our overall plan is to test the sensitivity and reliability of the micronucleus test used both in vitro and in vivo. This will include comparing the short term micronucleus test results with long term fates of animals as determined by classic toxicologic methods using the same animals for both kinds of determinations.

MOUSE LYMPHOMA CELL MUTATION TEST

INTRODUCTION

In the last two decades, serious concern about the possible genetic consequences of chemicals in our environment has been expressed. It has been estimated that 80-85% of human cancers could be caused by environmental factors (Hammond, 1975). In studying this problem it was demonstrated that there is a high correlation between mutagenic and carcinogenic activity (Malling, 1971; DeSerres, 1976). However, as observed by Miller and Miller (1976), even though a strong relationship exists between chemical mutagens and carcinogens, the causal relationship is not clear. Both mutagens and carcinogens cause heritable changes in phenotype (Pegg and Nicholl, 1976; Rajewsky and Goth, 1976). It has been suggested by Bridges (1976) that the relationship between mutagens and carcinogens may be in their "DNA damaging" ability. The existence of this relationship has led to a revival of the theory first proposed by Boveri in 1928 that cancer is a result of somatic mutation. The work discussed here is concerned with somatic mutation and not directly with carcinogenesis as such, but the correlation will be assumed.

There is a prime requirement to identify potential mutagens before they can damage the population at large. The tests described here concentrate on somatic mutation induction in mouse lymphoma cells in vitro. Mouse lymphoma cells have many advantages in their use as a mammalian mutagenicity assay system. The cells are very easy to handle and the tests are relatively fast, simple and cheap to do. The assays are very sensitive for a number of chemical mutagens. But their biggest advantage is that these mammalian cells can be directly used to determine basic mutagenic mechanisms.

Heritable effects can be detected in mammalian cells in culture using changes in gene function as the endpoint. The functions of different genes can be revealed by the use of selective agents. Examples of selective agents are azaguanine, thioguanine (TG), cytosine arabinoside (ara-C), thymidine (Tdr) and BrdU. These are all drugs which affect the purine or pyrimidine biosynthesis pathways. Cardiac glycosides like ouabain (Oua) which affect membrane permeability are also used as selective agents. Selection of mutated cells is based on the plating of large numbers of treated cells in medium containing the selective agent and scoring resistant mutant colonies after incubation. Four selective agents have been used in this study: TG, Tdr, Oua and ara-C.

The action of TG is mediated through the enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT). Cells become resistant by virtue of loss of the enzyme or a reduction in its activity (DeMars, 1974). HGPRT is concerned with the purine "scavenger" pathway in mammalian cells. The gene for HGPRT is located on the X-chromosome in mammalian cells, including human cells.

There are several postulated mechanisms of resistance to Tdr. These include altered transport across the cell membrane, increased pool size of deoxycytidine phosphates, or partial or complete loss of thymidine kinase activity (Anderson and Fox, 1974; Clive et al., 1972; Fischer, Lee and Calabresi, 1974). There is evidence for each of the possibilities.

Oua, a cardiac glycoside, is a specific inhibitor of the Na^+/K^+ activated ATPase which appears to be the enzyme responsible for the mammalian cell "sodium pump" which maintains a low internal sodium level and a high internal potassium level (Pestka, 1975). Although the requirement for the sodium pump is not understood, it is known that high potassium levels are required for a number of enzyme systems including protein synthesis. Oua binds to the phosphorylated enzyme and appears to prevent dephosphorylation (Tobin et al., 1974).

These three selective agents have all been used previously in a variety of mammalian cell systems to detect a range of known mutagens (Abbondandalo, 1977; Rogers, 1978). The use of ara-C as a selective agent was first reported by Fischer and coworkers (Fischer, Lee and Calabresi, 1974) but they provided little information on its use or the properties of ara-C resistant clones. Ara-C is a nucleoside analogue. Until recently its mode of action in cells was not completely understood. Incorporation assays (Graham and Whitmore, 1970) indicated that ara-C was mainly found in the acid soluble fraction of DNA. It had little effect on RNA or protein synthesis. Ara-C killed cells in S-phase of the cell cycle and temporarily blocked cells from passing from G_1 into S. It was known that ara-C had to be phosphorylated to ara-CTP before it became toxic (Graham and Whitmore, 1970). With the discovery of specific DNA polymerases in mammalian cells, it became possible to elucidate the mode of action of ara-C. DiCioccio and Srivistava (1978) found that ara-CTP inhibits DNA polymerases α and β by specifically competing with dCTP for binding. It also inhibits terminal transferase by competing with any substrate (dGTP, dCTP, dATP or TTP) for binding sites. Ara-CTP does not inhibit DNA polymerase γ.

Thus four different genetic endpoints can be examined in L5178Y mouse lymphoma cells. We have so far examined the effect of MMH, UDMH, and SDMH on L5178Y cells, and also EMS as a positive control.

MATERIALS AND METHODS

Two different growth media have been used in the experiments described here. In the initial series of experiments, the growth medium used was Fischer's medium (GIBCO), supplemented with sodium pyruvate (20 µg/ml, Sigma), penicillin/streptomycin (Sigma) and horse serum (GIBCO). However, some difficulty was encountered in treating the cells due to the high alkalinity caused by addition of hydrazine compounds to the growth medium. The growth medium was changed to McCoy's 5A medium (GIBCO) with the same supplements as listed above. McCoy's medium has twice the buffering capacity of Fischer's medium so it is able to maintain a more suitable pH level for adequate growth of the lymphoma cells when treated with hydrazines. A mutation experiment

was performed with EMS as the mutagen to determine if there are any differences in plating efficiency or mutation induction between the two types of cell media. No differences were found (see upper left portion of Figure 12 and Figure 14, respectively).

Toxicity experiments to determine the optimum treatment time and dose of test compound are carried out as follows: L5178Y mouse lymphoma cells are grown from low cell densities (10^2 - 10^3/ml) for 5 days prior to treatment. Cell suspensions are counted, centrifuged and resuspended if necessary, to give a cell density of 5 x 10^5 cells/ml. Test compound in 0.2 ml aliquots is added to 20 ml aliquots of cell suspension and incubated at 37° C in a humidified CO_2 incubator for 2 hr. For longer treatment periods up to 48 hr, the cells are set up at lower cell densities to allow for cell growth (i.e. 1 x 10^5 cells/ml for 24 hr treatment and 5 x 10^4 cells/ml for 48 hr treatment). All treatments are carried out in Fischer's or McCoy's medium supplemented with antibiotics and 10% horse serum (FM10 or M10). At the end of the treatment period, the cells are centrifuged and the supernatant discarded. The cells are then plated in Fischer's or McCoy's medium supplemented with antibiotics and 20% horse serum (FM20 or M20), solidified by agar and incubated for 10-12 days in a humidified CO_2 incubator to determine survival.

In a mutation experiment, the treatment regimen is as above. At the end of treatment, the test compound is removed, the cells washed twice in fresh medium and resuspended in 50 ml FM20 or M20. Each treatment level and the control are manipulated in the same way. The cells are counted and diluted to give 74.5 ml of 2 x 10^5 cells/ml. Six milliliters of this suspension is then added to 51 ml selective medium (FM20 or M20 plus selective agent, either Oua, TG, Tdr or ara-C) and solidified by adding 3 ml of 5% Noble agar (DIFCO). Eight plates are made per treatment with 7.5 ml/plate. For determination of survival, 0.5 ml of cell suspension is serially diluted to give a final concentration of 150 cells/plate (4 plates per treatment). To the remaining 50 ml of 2 x 10^5 cells/ml, 50 ml of FM10 or M10 is added. The cells are then incubated overnight in a humidified CO_2 incubator. Determination of mutation frequencies is made every 24 hr for 9 days. The plates for determination of induced mutation frequency and survival are incubated for 12 days at 37° C in a humidified CO_2 incubator.

In experiments with metabolic activation, the procedure is as follows: Cells are grown as detailed above and diluted to give a cell density of 5 x 10^5/ml. S9 mix (0.5 ml/20 ml cell suspension) and test compound (0.2 ml/20 ml cell suspension) are added and incubated at 37° C in a humidified CO_2 incubator for 4 hr. The S9 mix is made up as follows: 1 ml arochlor-1254 induced S9 (Litton), 0.5 ml NADP (5 mg/ml, Sigma), 0.5 ml glucose-6-phosphate (8 mg/ml, Sigma), and 1 ml Tris HCI (pH 7.5, Sigma).

RESULTS AND DISCUSSION

Toxicity

The results of experiments to determine the toxicity of EMS, MMH, UDMH and SDMH are shown in Figure 12. Skeleton toxicity experiments with EMS in Fischer's and McCoy's 5A cell medium are given here. More extensive toxicity data for EMS treatment of L5178Y mouse lymphoma cells are given in Rogers (1978). The experiment shown here was performed to compare the toxicity of EMS in the two cell media. It can be seen that EMS is identically toxic to cells in both cell media. Because of this result, we believe that all results using either cell medium are comparable, and no notation will be made of which medium was used in the experiments described.

Three different times were employed in testing the toxicity of the compounds. For mutation experiments a treatment time of 24 hr was adopted. Toxicity data were used to correct the mutation data for the number of mutants per the number of cells expected to survive at a particular dose level.

Experiments were performed to determine how long MMH and UDMH remain active while being incubated in McCoy's medium, including 10% horse serum. The chemicals in the medium were preincubated for 24 or 48 hr, the cells were added for 4 hr, and then they were plated to determine survival after removal of the test compound. The results are shown in Figure 13. The toxicity of MMH and UDMH after 24 or 48 hr preincubation appears comparable to the toxicity shown when the cells are present for the whole incubation period. Thus, either the compound is still present in the medium or its degradation products are equally toxic. However, another possibility may be that the pH change caused by the hydrazines may be sufficient to prevent the mouse lymphoma cells from growing normally. This possibility needs further investigation.

Mutation

EMS (2 hr treatment) produced a significant increase in the induced mutation frequency in all four selective systems (Figure 14). This result confirms those obtained previously with this cell system (Rogers, 1978; Rogers et al., 1979).

MMH in the presence or absence of metabolic activation did not produce a significant increase in the induced mutation frequency in any of the four selective systems used (Figures 15 and 16). Thus it appears that in L5178Y mouse lymphoma cells, MMH, although highly toxic, does not induce point mutations.

The results of mutation experiments with UDMH are shown in Figure 17. Significant mutation induction was noted only in the thymidine selective system. This result confirms those of Brusick and Mathesen

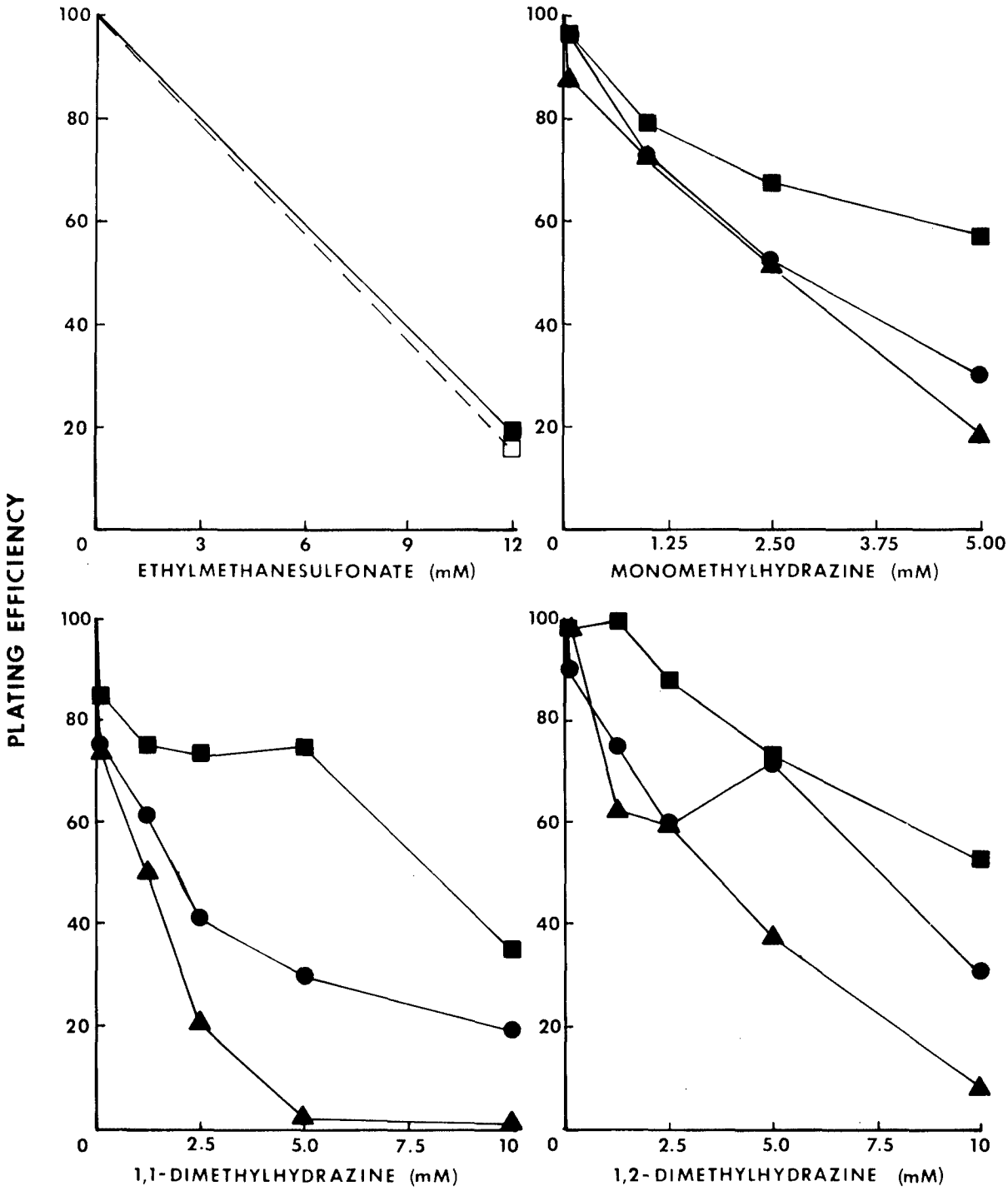


Figure 12. L5178Y mouse lymphoma cell survival after treatment with (upper left) EMS, (upper right) MMH, (lower left) UDMH, or (lower right) SDMH for (squares) 2 hr, (circles) 24 hr, or (triangles) 48 hr. In the upper left graph (EMS), the solid line and square are from data obtained using Fisher's cell medium and the dotted line and open square are from data obtained using McCoy's 5A cell medium.

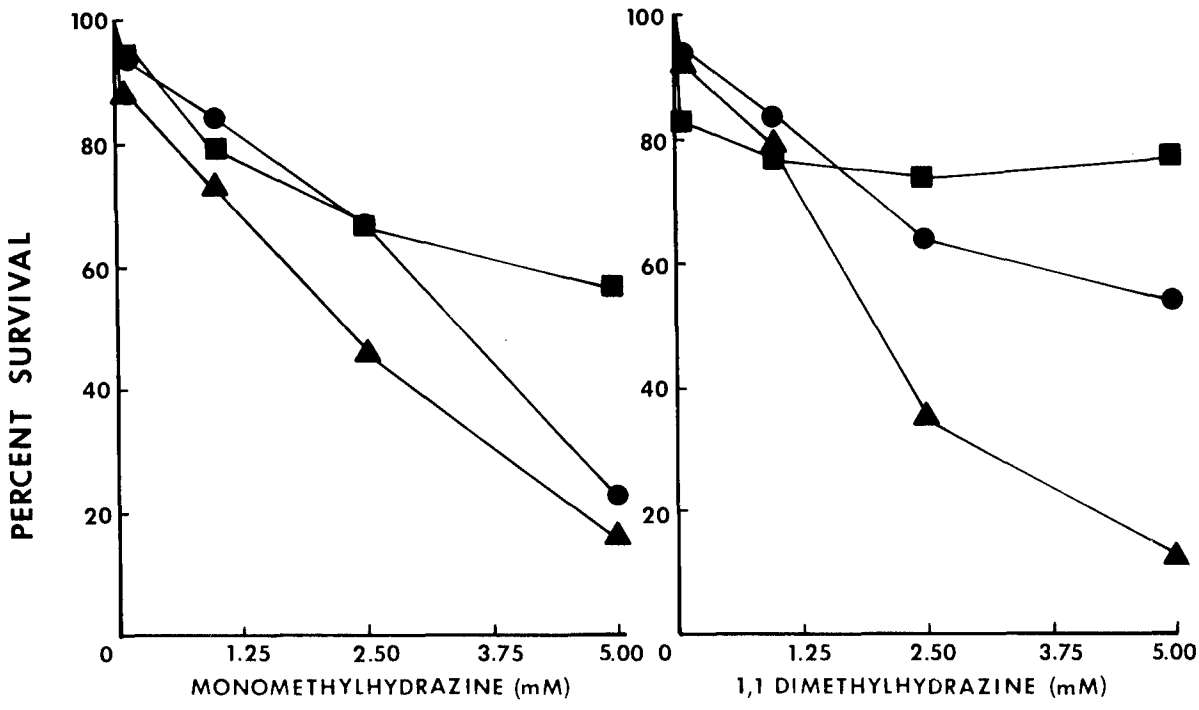


Figure 13. L5178Y mouse lymphoma cell survival after 2 hr treatment with (left) MMH, or (right) UDMH that had been preincubated in cell medium for (squares) 0 hr, (circles) 24 hr, or (triangles) 48 hr.

(1976). Experiments are presently underway to construct a dose response curve for UDMH induced Tdr resistance.

One mutation experiment with SDMH has been completed (Figure 18). The results indicate a weak positive response in the TG selective system. There is no significant increase in the induced mutation frequency in the Oua or ara-C selective systems. The result in the Tdr selective system is not clear since in the single experiment completed to date, the spontaneous mutation rate to Tdr resistance was abnormally high. A significant increase in mutation frequency in the treated cells was thus difficult to determine. Further experiments are planned to confirm these preliminary results with SDMH.

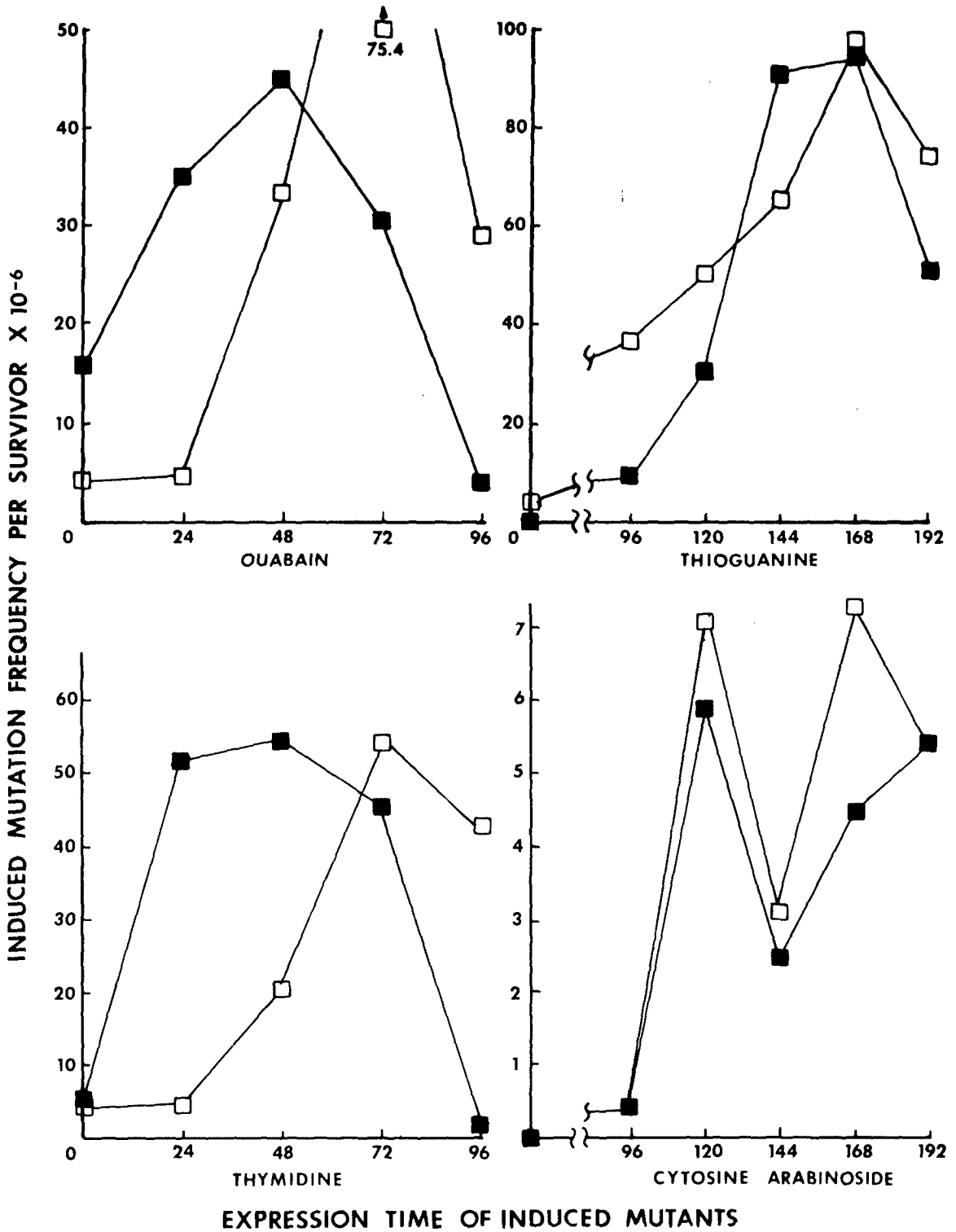


Figure 14. Induced (upper left) Oua, (upper right) TG, (lower left) Tdr, or (lower right) ara-C resistance mutation frequency (per survivor) in L5178Y mouse lymphoma cells treated for 2 hr with 1.2 x 10^{-2} M EMS. Cells grown in (solid squares) Fisher's cell medium or (open squares) McCoy's 5A cell medium. Control counts have been subtracted.

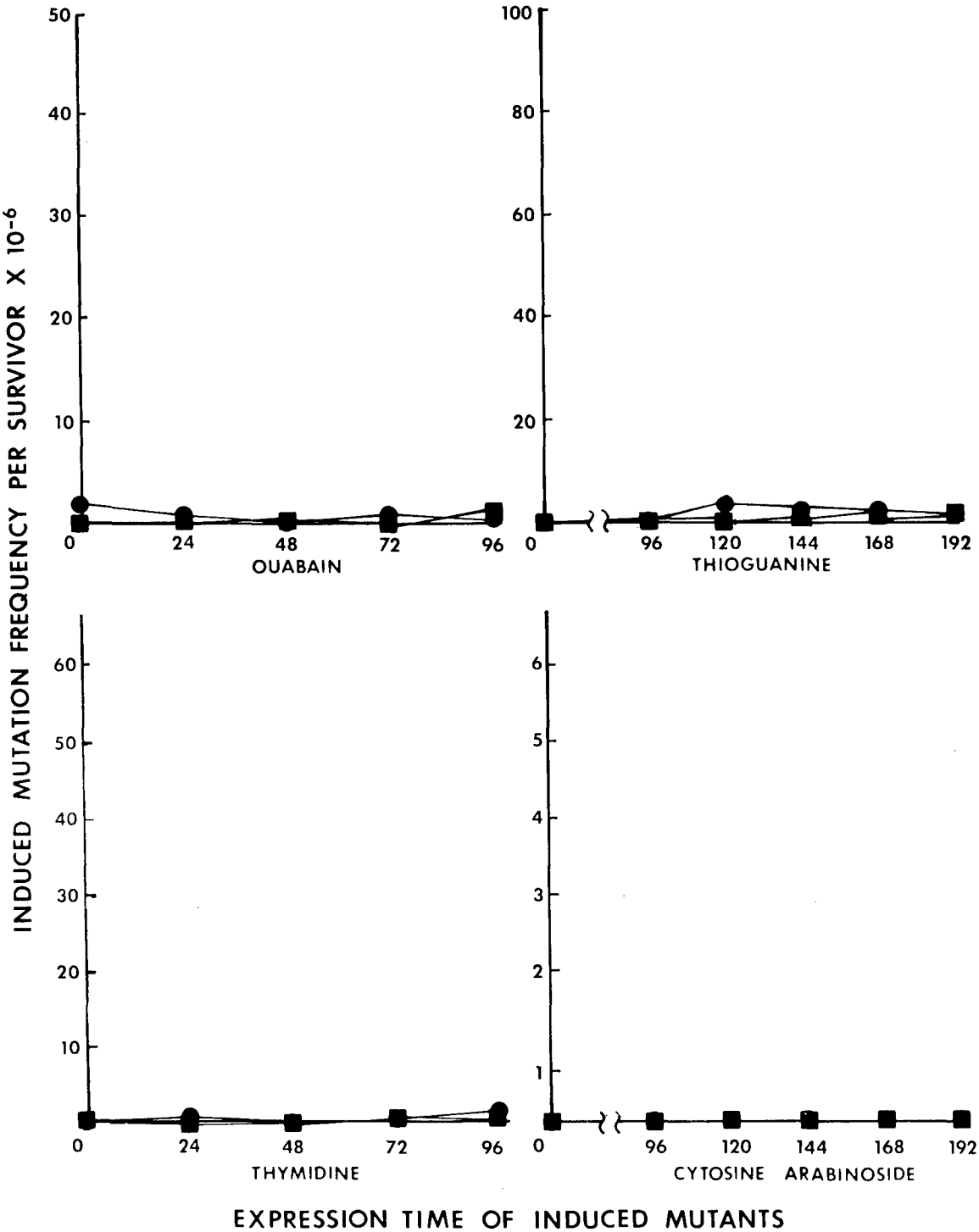


Figure 15. Induced (upper left) Oua, (upper right) TG, (lower left) Tdr, or (lower right) ara-C resistance mutation frequency (per survivor) in L5178Y mouse lymphoma cells treated for (squares) 24 hr with 2.5 mM MMH, or (circles) 2 hr with 5 mM MMH. Points are each averages of 2 and 3 experiments, respectively. Control counts have been subtracted.

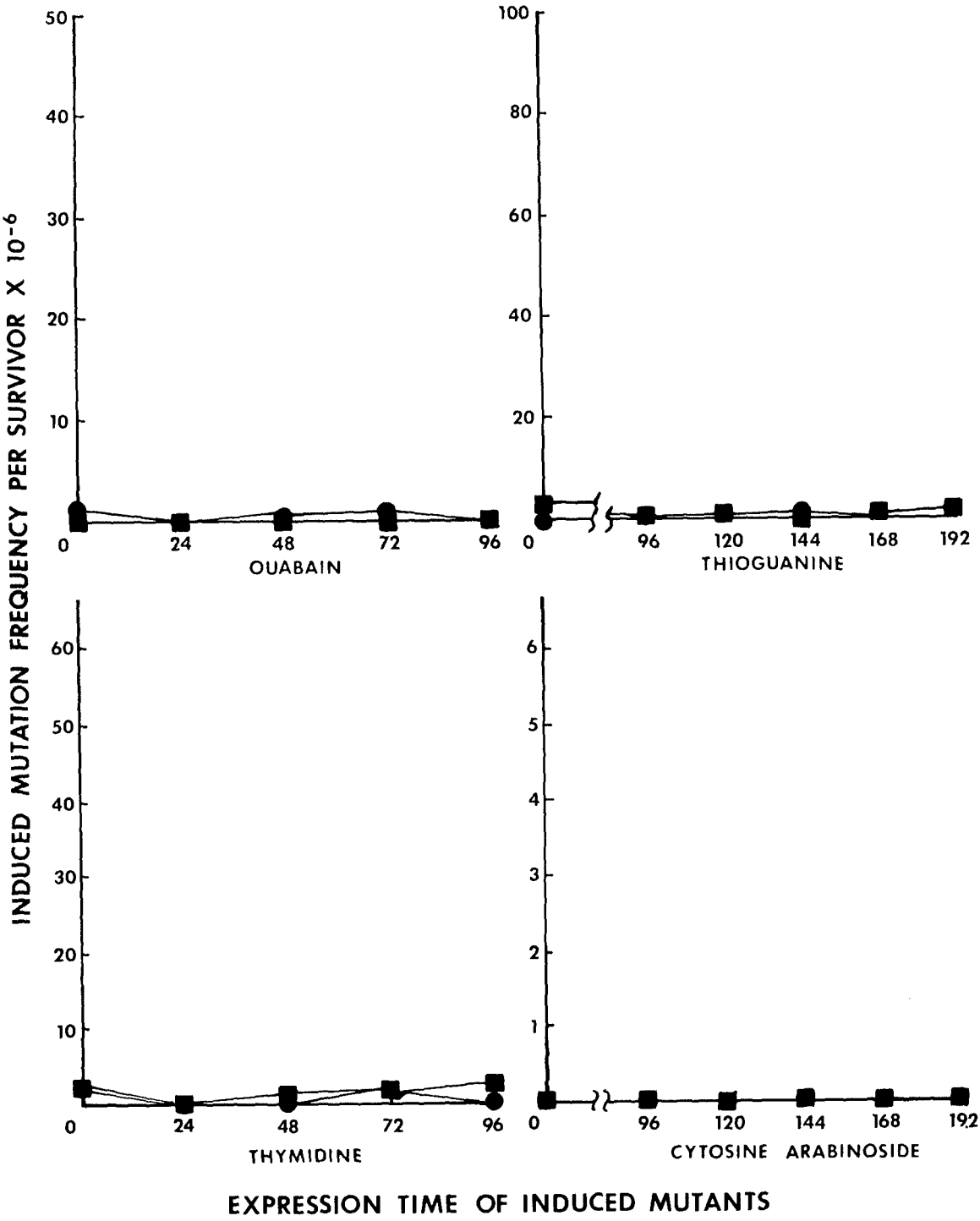


Figure 16. Induced (upper left) Oua, (upper right) TG, (lower left) Tdr, or (lower right) ara-C resistance mutation frequency (per survivor) in L5178Y mouse lymphoma cells treated for 4 hr with (squares) 1 mM or (circles) 2.5 mM MMH in the presence of activating liver enzymes. Points are each averages of two experiments. Control counts have been subtracted.

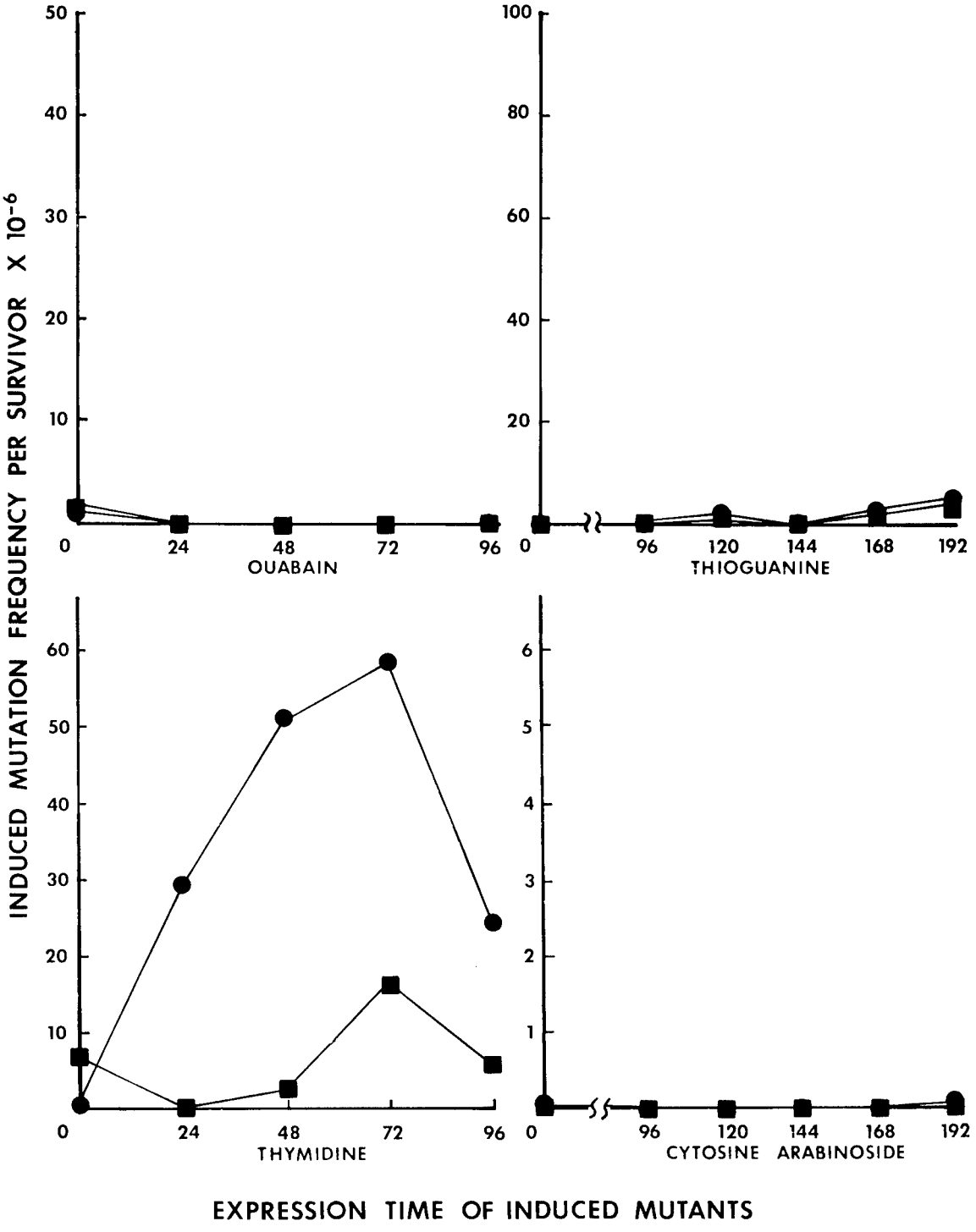


Figure 17. Induced (upper left) Oua, (upper right) TG, (lower left) Tdr, or (lower right) ara-C resistance mutation frequency (per survivor) in L5178Y mouse lymphoma cells treated for 24 hr with (squares) 2.5 mM or (circles) 5 mM UDMH. Points are each averages of two experiments. Control counts have been subtracted.

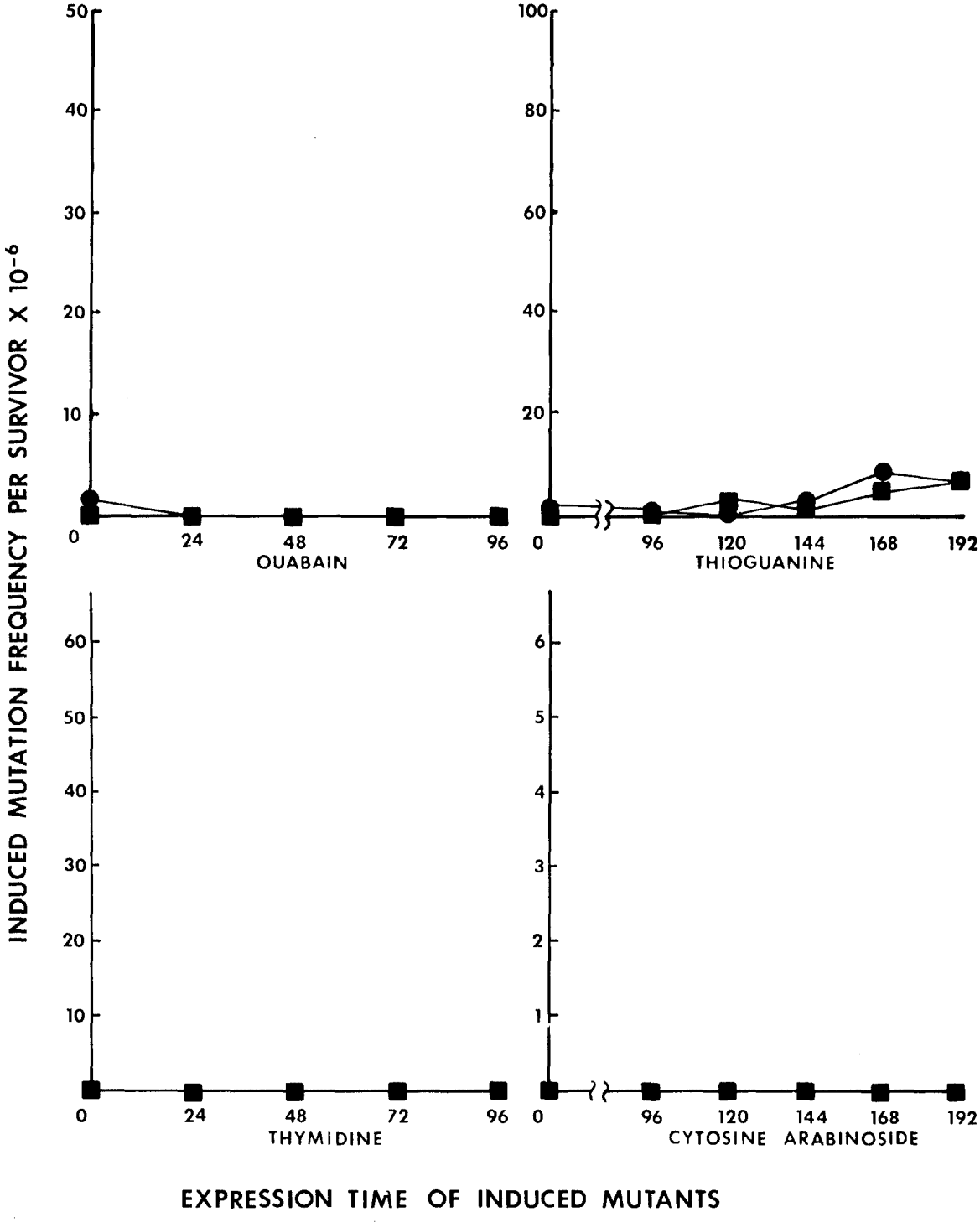


Figure 18. Induced (upper left) Oua, (upper right) TG, (lower left) Tdr, or (lower right) ara-C resistance mutation frequency (per survivor) in L5178Y mouse lymphoma cells treated for 24 hr with (squares) 2.5 mM or (circles) 5 mM SDMH. Control counts have been subtracted.

DNA REPLICATION AND CELL PROLIFERATION STUDIES

EFFECTS OF HYDRAZINE ON DNA REPLICATION IN CELL CULTURES

INTRODUCTION

In vitro tests have been done with animal cell cultures in order to determine whether hydrazine (Hz) may interact with DNA in such a way that DNA replication is inhibited. Recent studies by Painter (1977) have indicated that one of the first observable effects of mutagens and carcinogens in cell culture is an inhibition of normal DNA replication, presumably as the result of physical changes or chemical adducts which will not permit initiation of DNA replication or continuation of DNA replication past the point of DNA damage. This re- results in a progressive decline in the overall rate of DNA synthesis with time after a brief exposure of cells to the mutagen, which can be measured using radiotracer methods. Nonmutagenic toxins (e.g. hydroxyurea, cycloheximide) inhibit DNA replication, but the rate of DNA synthesis returns to normal upon removal of the toxin. Thus, it has been proposed that a test for mutagenic or carcinogenic activity of a material may be its ability to induce irreversible (or only very slowly reversible as DNA repair occurs) inhibition of DNA synthesis when cells are exposed to the material for a relatively brief period. A number of known mutagens have been shown to inhibit DNA synthesis in the manner described. These include various alkylating agents, UV light, and benzo(a)pyrene. The latter compound must first be converted to its active form by rat liver enzymes in order to be active in the test.

In this laboratory, the above test has been applied to Hz using four cell lines. They are Chinese hamster ovary (CHO), Chinese hamster lung fibroblasts (V-79), and two cell lines established in this laboratory from adult rat lung (ARL-12 and ARL-14). The latter cell lines have some of the characteristics of alveolar type II cells.

MATERIALS AND METHODS

Following the method described by Painter (1977), replicating cell cultures were prelabeled with ^{14}C-thymidine (^{14}C-Tdr) for at least one cell cycle in order to uniformly label the DNA of the culture and thus provide a method for estimation of the amount of DNA present during subsequent manipulations. Labeled cultures were then exposed to various concentrations of Hz dissolved in phosphate buffered saline (PBS, pH 7.0). Methylmethanesulfonate (MMS) at 10^{-3} M, a known mutagen, was used as a positive control. Concentrations of Hz were measured at the beginning and end of the Hz exposures using a colorimetric assay (Malone, 1970). There was no apparent degradation of Hz during the cell exposures, or even when the Hz solutions were stored overnight in the refrigerator.

After a 1 hr exposure to Hz or MMS, the cultures were returned to normal growth medium. Control cultures were exposed for a like period to PBS. Immediately following the 1 hr exposure, and at selected intervals later, sample cultures were pulse labeled with ^{3}H-Tdr for 10 min. The doubly labeled cells were then quickly scraped from the culture dishes and collected on glass fiber filters where they were washed with cold 5% trichloroacetic acid and alcohol to remove any unbound label. After drying, the filters were counted by liquid scintillation spectrometry. The ratio of ^{3}H DPM/^{14}C DPM was calculated which then gave a measure of the rate of DNA synthesis at the time of the pulse labeling with ^{3}H-Tdr.

RESULTS AND DISCUSSION

Tables 2 - 5 present the results obtained when DNA synthesis was measured at 1 and 4 hr following treatment with various concentrations of Hz.

Table 2. Effect of hydrazine on DNA synthesis in CHO cells.

Experiment Number 1			Experiment Number 2		
Hydrazine Concentration	Percentage of Control		Hydrazine Concentration	Percentage of Control	
	1 hr	4 hr		1 hr	4 hr
3.0×10^{-4} M	128	95.6	3.9×10^{-3} M	44.3	33.6
1.5×10^{-3} M	111	72.2	7.8×10^{-3} M	25.7	18.6
3.0×10^{-3} M	93.9	61.5	1.5×10^{-2} M	4.86	2.52
1.5×10^{-2} M	24.8	15.9	MMS 10^{-3} M	39.0	3.38
3.0×10^{-2} M	24.5	7.4			

Table 3. Effect of hydrazine on DNA synthesis in V-79 cells.

Experiment Number 1			Experiment Number 2		
Hydrazine Concentration	Percentage of Control		Hydrazine Concentration	Percentage of Control	
	1 hr	4 hr		1 hr	4 hr
7.8×10^{-3} M	23.9	16.3	3.9×10^{-3} M	42.3	55.7
1.5×10^{-2} M	4.74	1.35	7.8×10^{-3} M	39.9	47.7
3.1×10^{-2} M	2.53	0.92	1.5×10^{-2} M	13.09	1.14
MMS 10^{-3} M	61.1	5.98	MMS 10^{-3} M	63.2	1.85

Table 4. Effect of hydrazine on DNA synthesis in ARL-12 cells.

Hydrazine Concentration	Experiment Number 1 Percentage of Control		Experiment Number 2 Percentage of Control		Experiment Number 3 Percentage of Control	
	1 hr	4 hr	1 hr	4 hr	1 hr	4 hr
3.0×10^{-4} M	60.2	57.2				
1.5×10^{-3} M	84.6	66.7				
3.0×10^{-3} M	138.2	83.7				
3.9×10^{-3} M					34.9	20.6
7.8×10^{-3} M			21.6	9.9	13.3	30.0
1.5×10^{-2} M	76.0	40.1	0.99	0.73	17.3	28.6
3.0×10^{-2} M	4.2	3.9	1.5	0.88		
MMS 10^{-3} M			73.1	13.1	46.3	28.3

Table 5. Effect of hydrazine on DNA synthesis in ARL-14 cells.

Hydrazine Concentration	Experiment Number 1 Percentage of Control		Experiment Number 2 Percentage of Control		Experiment Number 3 Percentage of Control	
	1 hr	4 hr	1 hr	4 hr	1 hr	4 hr
3.0×10^{-4} M	75.0	55.9				
1.5×10^{-3} M	75.1	64.8				
3.0×10^{-3} M	118.2	77.9				
3.9×10^{-3} M					57.9	48.9
7.8×10^{-3} M			11.3	6.8	4.7	4.7
1.5×10^{-2} M	87.9	61.1	2.9	1.2	1.7	8.1
3.0×10^{-2} M	10.7	9.1	1.6	2.2		
MMS 10^{-3} M			41.5	14.5	26.7	13.5

The results are presented in the tables as percentages of untreated controls, and are based on triplicate dishes in all cases. The positive control, MMS, caused the expected effect in all cell lines, with a reduction in the rate of DNA synthesis at the time of exposure, and a much lower rate at 4 hr postexposure. With Hz, there was a clear dose response effect, with only a slight effect

on DNA synthesis until concentrations of 0.25 - 0.50 mg/ml (0.78 - 1.5 x 10^{-2}M) were reached, at which point there was a sharp reduction in the rate of DNA synthesis. Four hours later, the rate of synthesis was little different and in most cases was even lower. These results suggested that Hz behaves something like known mutagens in regard to its inhibitory effect on DNA synthesis.

A second series of experiments was done in order to follow the effect of Hz on the rate of DNA synthesis with time after a single exposure. The concentration of Hz was 1.5 x 10^{-2} M and of MMH was 10^{-3} M. The results are shown in Figure 19. The response to MMS was similar in all cell lines during the first few hours. By 24 hr, the rate of DNA synthesis in lines V-79 and CHO had returned to near normal, but in the two rat ling cell lines, this did not occur. In all cell lines, Hz produced an effect qualitatively similar to that of MMS; however, the ARL cell lines were much more affected than either V-79 or CHO. At 24 hr post Hz treatment, DNA synthesis in line V-79 had returned to normal, but only a slight recovery was seen in the other cell lines.

These studies have suggested that Hz may interact directly with cellular DNA, and therefore could possibly have mutagenic effects under some conditions.

EFFECT OF INHALATION OF JP-5 ON CELL PROLIFERATION IN MICE AND RATS

INTRODUCTION

In November, 1977 a group of animals (rats and mice) that had been exposed by inhalation to JP-5 at the THRU was shipped to UCI for study. The object was to determine whether the inhalation exposure had produced an increase in cellular proliferative activity in selected organs.

MATERIALS AND METHODS

The animals used in this study were all female C57Bl/6 mice and Fisher 344 rats. Three of each were held as shelf controls, exposed to 0.15 mg/l JP-5, or exposed to 0.75 mg/l JP-5 (MacEwen and Vernot, 1978). The exposures were continuous for 90 days.

Upon arrival at UCI, the animals were injected with ^{3}H-Tdr (100 μCi/mouse, 200 μCi/rat). After 1 hr, the animals were killed by an overdose of sodium pentobarbital, and selected organs were removed, fixed, embedded, and sectioned for autoradiography. Sections of 6 μm thickness were mounted on glass slides and dipped in Eastman NTB-2 diluted 1:1 with water. After 2 months exposure at -20° C, the slides were developed and stained with H & E. The fraction of labeled cells in lung, liver, and kidney was determined by counting at least 1000 cells per section.

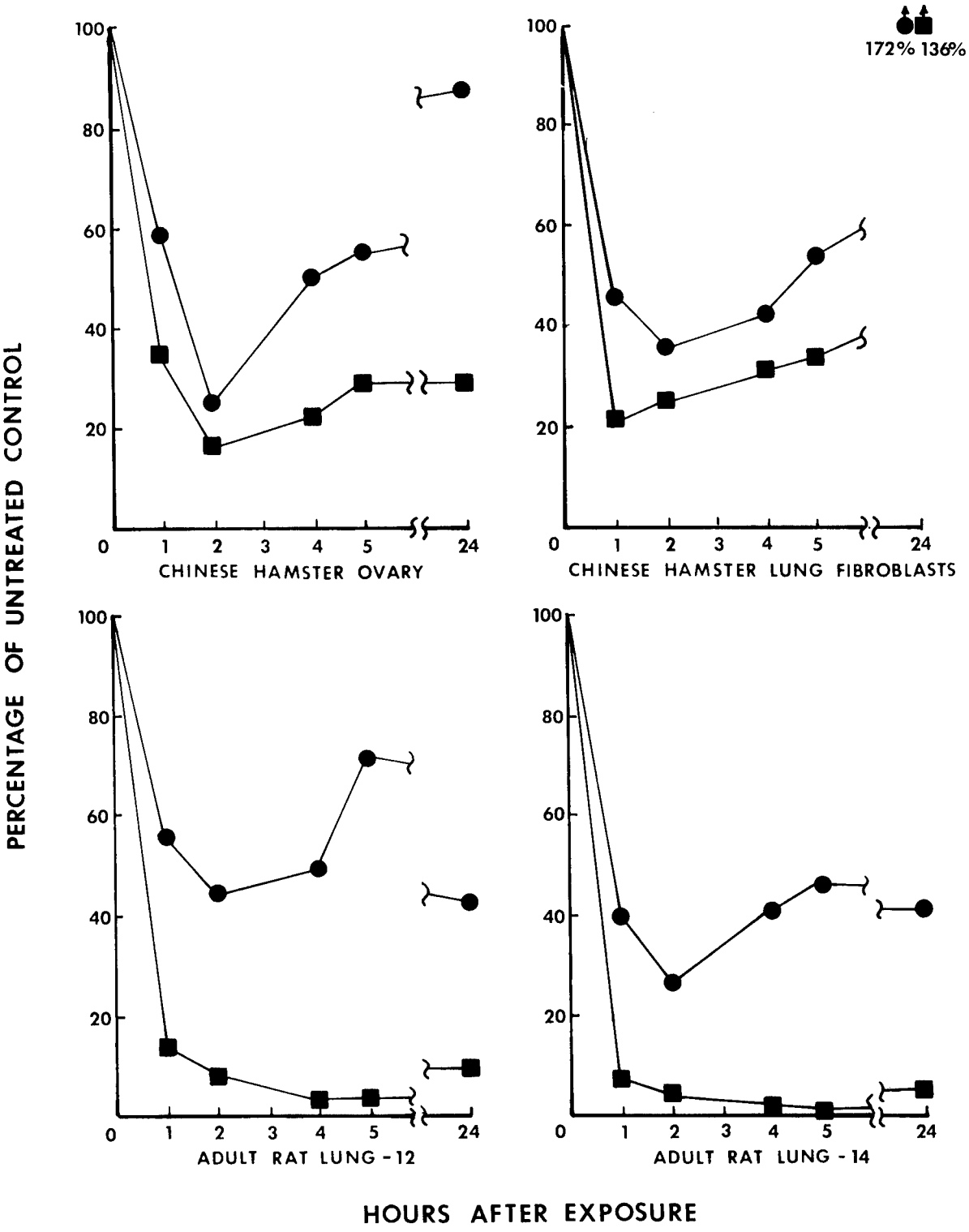


Figure 19. DNA synthesis (as percentage of control) in (upper left) CHO, (upper right) V-79, (lower left) ARL-12, or (lower right) ARL-14 cells exposed to (squares) 10^{-2} M MMH or (circles) 1.5 x 10^{-3} M MMS for 1 hr.

RESULTS AND DISCUSSION

The results of this experiment are shown in Table 6. This study did not give any indication of a stimulation of cellular proliferation by the exposure to JP-5 in the organs studied. One of the control mice had an abdominal tumor which had invaded the liver and other organs. In this animal, there was an increase in the labeling index in the liver.

Table 6. ^{3}H-Thymidine labeling index in tissues of rats and mice exposed to JP-5*

Tissue	Control	0.15 mg/ml	0.75 mg/ml
Rat Lung	0.017; 0.010	0.007	0.017; 0.001
Rat Liver	0.020; 0.017	0.038	0.015; 0.014
Rat Kidney	0.001; 0.001	0.001	0.004; 0.007
Mouse Lung	0.010; 0.013; 0.009**	0.006; 0.002	0.003; 0.010
Mouse Liver	0.015; 0.007; 0.028**	0.006	0.004; 0.012
Mouse Kidney	0.010; 0.009	0.002; 0.004	0.008; 0.003

* Values are the fraction of labeled cell nuclei, based on counts of 1000 cells, and represent individual animals.

** This animal had an abdominal tumor of undetermined cell type.

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